Adipocytes Differentiated In Vitro from Rat Mesenchymal Stem Cells Lack Essential Free Fatty Acids Compared to Adult Adipocytes

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Adult bone marrow mesenchymal stem cells (BMSCs) can be differentiated in vitro to become adipocyte-like cells with lipid vacuoles, similar to adipocytes derived from adult adipose tissue. Little is known regarding the composition of free fatty acids (FFAs) of the in vitro-differentiated adipocytes, or whether it resembles that of native adult adipocytes. We used gas chromatography-mass spectrometry to identify FFA species in BMSC-derived adipocytes and compared them with FFAs found in adipocytes derived from adult adipose tissue. We found that adult adipocytes contained significant percentages of saturated and monounsaturated FFAs, including palmitic acid (C16:0), stearic acid (C18:0), and oleic acid (C18:1); some polyunsaturated FFAs, such as linoleic acid (C18:2), a small percentage of arachidonic acid (C20:4), and very little linolenic acid (C18:3). In comparison, 80%–90% confluent BMSCs contained comparable percentages of palmitic and oleic acids, significantly more arachidonic and stearic acids, very little linoleic acid, and no linolenic acid. After differentiation, compared with adult adipocytes, BMSC-derived adipocytes contained a comparable percentage of palmitic acid, more stearic and arachidonic acids, less oleic acid, almost no linoleic acid, and no detectable linolenic acid. This composition was quite similar to that of undifferentiated BMSCs. The differentiation medium contained only palmitic and stearic acids, with traces of oleic acid; it did not contain the essential polyunsaturated fatty acids. Thus, the composition of FFAs in BMSC-derived adipocytes was altered compared with adult adipocytes. BMSC-derived adipocytes had an altered composition of saturated and monounsaturated FFAs and lacked essential FFAs that may directly affect signaling related to their lipolysis/lipogenesis functions.

Introduction

ADULT BONE MARROW MESENCHYMAL stem cells (BMSCs) can be differentiated into adipocytes according to a well-established protocol [1]. Despite the similarities between adult cells and in vitro-differentiated stem cells, on transplantation in vivo, many cells obtained in vitro fail to perform according to their phenotype. Thus, in vitro-obtained cells may prove to have significant structural and functional shortcomings, compared with their adult counterparts. For example, in vitro differentiated mesenchymal stem cells from the synovial membrane failed to form stable cartilage in vivo [2]; transplanted BMSCs did not differentiate into neural cells in vivo [3]; BMSCs that could be differentiated in vitro to de novo insulin-producing cells did not cure diabetes after transplantation [4]. Moreover, BMSCs failed to transdifferentiate into adipocytes after implantation in adult adipose tissues in mice [5]. However, when exposed in culture to a proper combina-

tion of factors, BMSCs could differentiate and accumulate intracellular lipid vacuoles that stained positively for Oil Red O, similar to native adult adipocytes [1]. The differentiation process takes about 3 weeks; during that time, the stem cells round up, accumulate intracellular lipids, and acquire the overall morphology of an adipocyte. Although there is ample evidence that BMSC-derived adipocytes share numerous properties with native adult adipocytes, few investigations have focused on the differences between these cells. This may be necessary to understand why in vitro-differentiated adipocytes sometimes fail to behave in vivo similar to their native adult counterparts.

Adult adipocytes depend on free fatty acids (FFAs) to perform some of their specific functions [6]. In primary cultures of human adipocytes, polyunsaturated fatty acids (PUFAs) were shown to regulate the expression of lipid metabolism genes [7] and antagonize ligand-dependent peroxisome proliferator-activated receptor gamma (PPAR γ)

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activity [8]. Conjugated linoleic acid induced human adipocyte delipidation [9]. PEGylated linoleic acid stimulated lipolysis in 3T3-L1 cells [10] and activated the integrated stress response pathway in adipocytes [11]. When administered in a diet, linoleic acid reduced adipocyte size and changed the secretion of adipokines in obese rats [12]; in rat adipocytes linoleic acid influenced the secretion of leptin and adiponectin and its dietary intake reduced body fat in animals and some humans [13]. Given the important role that FFAs play in adipocyte functions, we hypothesized that, for BMSC-derived adipocytes to be functionally and structurally equivalent to adult adipocytes, they should have a comparable FFA composition, among other features. However, little data are available regarding the FFA composition of adipocytes to enable such evaluations of functional equivalence.

This study aimed at comparing the FFA composition in BMSC-derived adipocytes with the FFA composition found in freshly prepared adult adipocytes from inguinal fat pad of normal rats. Mesenchymal stem cells were harvested from rat femur, according to the procedure described in [14] and cultured to generate adipocytes, as described in [1,15]. The FFA composition was investigated qualitatively by gas chromatography-mass spectrometry.

Materials and Methods

Animals

All animal experiments complied with the guidelines of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, France, 1986). The experimental protocol was approved by the University of Medicine and Pharmacy Timisoara Ethical Committee.

Sprague-Dawley rats that weighed 200–250 g were housed in controlled temperature (23°C), with a 12-h light/12-h dark cycle, and with *ad libitum* access to standard laboratory rat chow (Rat Diet, Cantacuzino Institute, Bucharest, Romania) and water. All reagents were purchased from Sigma-Aldrich Company (Ayrshire, United Kingdom), unless otherwise stated.

Cell isolation and cultures

Normal rat BMSCs were obtained from adult male Sprague-Dawley rats by drilling into the femoral bone and extracting bone marrow [14]. About 1 mL of bone marrow material was harvested from each rat. The bone marrow was mixed with culture medium and placed in T75 culture flasks. After 48 h, the nonadherent fraction was discarded, fresh medium was added to the plates, and the adherent BMSCs were further cultured. Cells were expanded in α minimum essential medium (Gibco BRL, Invitrogen, Carlsbad, CA), supplemented with 10% fetal calf serum (FCS; PromoCell, Heidelberg, Germany) and 2% penicillin/streptomycin (Pen/Strep, 10,000 IU/mL; PromoCell). Cells were incubated at 37°C in 5% CO₂, and medium was replaced every 3 days. On days 8–9, the BMSCs reached 80%–90% confluence. Confluent cells were passaged by adding 0.25% Trypsin-EDTA solution (Sigma-Aldrich Company), then centrifuging them for 10 min at 300 g, and replating them in 3 T75 culture flasks at a density of 10⁴ cells/cm². Cells again reached 80%–90% confluence after about 4–5

days. After a second trypsinization, the cells from each T75 culture flask were split in 2 groups, as follows:

- Most cells were placed into 2 T75 culture flasks and, after a few days, when they reached 80%–90% confluence, the cells were subjected to the adipogenesis differentiation protocol. After the differentiation protocol, the cells from all flasks were combined and processed for FFA derivatization;
- A small amount of cells were placed on 2-well chamber glass slides and, when they reached 80%–90% confluence, they were subjected to the adipogenesis differentiation protocol, as described next. At different incubation times, they provided BMSCs at 80%–90% confluence, BMSC-derived preadipocytes, and BMSC-derived adipocytes that were used for Oil Red O staining;

For the next 3 weeks, the BMSCs were stimulated to differentiate in adipogenic medium that contained Dulbecco's modified Eagle's medium (DMEM), 10% FCS, 45 mM 3-isobutyl-1-methylxanthine, 50 μ M indomethacin, 10 mg/mL insulin, and 10 mM hydrocortisone, supplemented with 1% Pen/Strep. After a week of exposure to the differentiation protocol, we harvested preadipocytes; after 2 more weeks, we harvested fully differentiated adipocytes. We monitored the *in vitro* development of BMSCs into preadipocytes and then into differentiated adipocytes via immunohistochemistry by determining the expression of fatty acid binding protein 4, and by reverse transcription-polymerase chain reaction, evaluating the mRNA levels for the genes of peroxisome proliferator-activated receptor gamma (PPAR γ), lipoprotein lipase (LPL), and CCAAT/enhancer binding protein alpha (C/EBP α), as previously described [15].

Gas chromatograph-mass spectrometry of FFAs

The BMSC-derived cells were harvested, washed twice in phosphate-buffered saline (PBS), and resuspended in 200 μ L cold lysis buffer (150 mM NaCl, 20 mM Tris-HCl, 0.2% Triton-X 100 pH=7.4). An adipose tissue sample was prepared from a fragment of adipose tissue (~50 mg) that was homogenized in 500 μ L cold PBS. Freshly isolated adipocytes were prepared from another sample of adipose tissue (about 50 mg), that was cut into small pieces and digested with collagenase type IV-S in DMEM for 2 h at 37°C. These cells were then washed twice with PBS and resuspended in 200 μ L cold lysis buffer. All cell lysates and the tissue homogenate were subjected to derivatization to convert the FFAs to fatty acid methyl esters, as previously described [16]. Briefly, a 150 μ L aliquot of each cell lysate or homogenate was added to a 5 mL mixture of 50:1 v/v methanol:acetyl chloride in a screw-top glass tube. The tubes were vortexed at room temperature in the dark for 45 min, and the methylation was stopped by adding 3 mL of 0.25 M potassium carbonate. After 5 min, 1 mL hexane was added, and the tubes were vigorously vortexed in the dark for 1 h at room temperature to extract the FFA esters. The tubes were left to stand for a few minutes to allow separation of the aqueous and organic phases. The upper, hexane layer, which contained the FFA methyl esters, was collected and evaporated under nitrogen. The dry residue was redissolved in 150 μ L of hexane, and a 2 μ L aliquot was injected into a HP6890 Series Gas Chromatograph coupled with a Hewlett Packard 5973 Mass

Selective Detector. The gas chromatograph was equipped with a split-splitless injector and a Factor Four™ Capillary Column VF-35 ms, (30 m long and 0.25 mm inner diameter), with a film thickness of 0.25 μ m. For gas chromatography, the temperature ranged from 100°C to 300°C, with a 6°C/min gradient, and the solvent delay was set at 7 min. The injector was maintained at 250°C, and helium was the GC carrier gas. The flow was set at 1.0 mL/min, and the sample was injected in the splitless mode. The mass spectrometry conditions were the following: ionization energy, 70 eV; electronic impact ion source temperature, 200°C; quadrupole temperature, 100°C; scan rate, 1.6 scan/s; mass, 40–500 amu [17]. For the identification of compounds, the mass spectra of the samples were compared with mass spectra in the Mass Spectral Library 2.0 developed by the National Institute of Standards and Technology/Environmental Protection Agency/National Institutes of Health. The retention times for derivatized FFAs were as follows:

- palmitic acid (C16:0) 18.26 min;
- oleic acid (C18:1) 21.29 min;
- stearic acid (C18:0) 21.39 min;
- linoleic acid (C18:2) 21.45 min;
- linolenic acid (C18:3) 21.65 min;
- arachidonic acid (C20:4) 24.19 min;

The peak areas of these 6 FFAs summed to 100%, and the percentage of area that corresponded to each FFA was plotted.

Oil Red O staining

When BMSCs reached 80%–90% confluence, they were stained with Oil Red O. Preadipocytes and adipocytes obtained in vitro were stained with Oil Red O on the 7th and 21st day of differentiation, respectively. Adult adipocytes obtained from adipose tissue digested with collagenase type IV-S (from *Clostridium histolyticum*) were washed twice with 1% bovine serum albumin-PBS, then cytospun for 6 min at 600 rpm. Before Oil Red O staining, slides were dried under an air flow for 5 min. The Oil Red O working solution was prepared by mixing 15 mL of a stock solution (0.5% Oil Red O in 60% isopropanol) with 10 mL of distilled water and filtering through a polyvinylidene fluoride membrane filter (0.22 μ m). Briefly, cells were washed twice with PBS, fixed in 10% formaldehyde in phosphate buffer for 1 h at room temperature, then washed with 60% isopropanol, and stained for 10 min with Oil Red O solution. Then, slides were rinsed several times in tap water, and the nuclei were counter-stained by immersion for 10 s in hematoxylin solution (Hematoxylin, Mayer's Lillie's Modification, Dako, Glostrup, Denmark).

Statistical analysis

All data are displayed as mean $\pm$ SE from at least 3 independent experiments. Statistical calculations were performed with the Gnumeric Spreadsheet (Gnome Office Suite, Gnome Foundation, Cambridge, MA). Comparisons between groups were performed with the Student's *t*-test, and values of $P < 0.05$ were considered significant.

Results and Discussion

In 3- μ m cryosections of whole adipose tissue samples, we detected the typical Oil Red O positivity indicative of lipid

accumulation (Fig. 1A). Adult adipocytes isolated from adipose tissue also stained positive for Oil Red O (Fig. 1B). This indicated that both adipose tissue and isolated adipocytes contained intracellular lipid vacuoles of variable sizes. Next, we examined rat BMSCs, before (Fig. 1C) and after differentiation in vitro under specific culture conditions to preadipocytes (Fig. 1D) and then to adipocyte-like cells (Fig. 1E). Some BMSC-derived preadipocytes and most adipocytes (Fig. 1D, E) also contained intracellular lipid vacuoles that stained positive for Oil Red O, indicating that BMSC could differentiate in vitro to adipocyte-like cells. Next, we analyzed the FFAs present in the adipose tissue, adult adipocytes, BMSCs, BMSC-derived preadipocytes, and BMSC-derived adipocytes. In adult rat adipose tissue, we identified 6 types of FFAs. The largest fraction comprised saturated FFAs, like palmitic and stearic acids; there were also significant percentages of oleic and linoleic acids; we found only small amounts of arachidonic acid and very small quantities of linolenic acid (Fig. 2A, first column in each group). When maintained on a constant diet, the FFA composition of the adipose tissue of rats was relatively constant (data not shown). This FFA composition was largely maintained in the isolated adult adipocytes (Fig. 2A, second column in each group). The only exceptions were stearic acid, which appeared to comprise a higher percentage in isolated adipocytes than that observed in whole adipose tissue, and linolenic acid, which was absent from the isolated adipocytes. These differences may have reflected the loss of adipocytes during isolation, the small number of isolated adipocytes, and the fact that collagenase treatment released stromal cells, which were harvested with adipocytes in the isolation procedure.

Compared with adipose tissue and isolated adipocytes, the rat BMSCs at 80%–90% confluence contained comparable percentages of palmitic and oleic acids, a significantly larger percentage of arachidonic and stearic acids, very small amounts of linoleic acid, and virtually no linolenic acid (Fig. 2A, third column in each group). Again, compared with adipose tissue, BMSC-derived preadipocytes contained comparable percentages of palmitic, oleic, and arachidonic acids, significantly higher percentages of stearic acid, barely detectable linoleic acid, and undetectable linolenic acid (Fig. 2A, fourth column in each group). Compared with adipose tissue, BMSC-derived adipocytes contained comparable percentages of palmitic acid, a higher percentage of stearic and arachidonic acids, a lower percentage of oleic acid, almost no linoleic acid, and no detectable linolenic acid (Fig. 2A, fifth column in each group). These results suggest that BMSC-derived adipocytes have a different FFA composition than the adult adipocytes.

Specific FFA chromatograms of adipose tissue, adult adipocytes, BMSCs, BMSC-derived preadipocytes, and BMSC-derived adipocytes are shown in Fig. 2B–F.

We analyzed next the adipocyte differentiation medium for FFA content and found that it contained only small amounts of palmitic and stearic acids, with traces of oleic acid. These were probably present in the serum added to the differentiation medium. We found no linoleic, linolenic, or arachidonic acid (Fig. 2G). Thus, it was not surprising that the BMSC-derived adipocytes lacked linolenic acid and contained negligible amounts of linoleic acid, 2 PUFAs that are essential in adipocyte function.

FIG. 1. Differentiation of adipocytes from BMSCs. All sections were stained with Oil Red O, and the nuclei were counter-stained with hematoxylin. **(A)** Adult adipose tissue showed scattered Oil Red O positivity. **(B)** Adult adipocytes, isolated from the adipose tissue are also Oil Red O positive. **(C)** BMSCs at 80%–90% confluence. Oil Red O staining was absent, because these cells had not begun to accumulate intracellular lipids. **(D)** BMSC-derived preadipocytes. Isolated cells began to accumulate intracellular lipids. **(E)** BMSC-derived adipocytes. Most cells have intracellular vacuoles of lipids that stain intensely red with Oil Red O. Magnification is 40 $\times$. BMSC, bone marrow mesenchymal stem cell. Color images available online at www.liebertonline.com/scd

We also noted additional peaks in some chromatograms (at 18.66 min, for instance). Those peaks represented compounds identified with a low probability (<50%) as 1,1-dimethoxydecane and phthalate derivatives. They were unrelated to FFAs, but could leak from the plastic labware. These peaks were absent in the chromatogram of the culture medium that was not exposed to culture plates (Fig. 2G). They formed small peaks in the chromatograms of adipose

tissue and the isolated adult adipocytes, which were handled only briefly in the plasticware. However, they were more obvious in chromatograms of the cultured cells; this suggested that indeed, these chemicals leaked from the walls of the culture plates and were processed during the derivatization procedure.

In conclusion, we found that BMSC-derived adipocytes differed from their adult *in vivo* counterparts with regard to

FIG. 2. Gas chromatography-mass spectrometry of FFAs in rat adipose tissues and BMSC-derived adipocytes. **(A)** FFA composition of whole adipose tissue (column 1), isolated adult adipocytes (column 2), BMSCs (column 3), BMSC-derived preadipocytes (column 4), and BMSC-derived adipocytes (column 5). **(B)** chromatogram of FFA composition of rat adipose tissue. **(C)** chromatogram of FFA composition of adult adipocytes isolated from rat adipose tissue. **(D)** chromatogram of FFA composition of cultured rat BMSCs. **(E)** chromatogram of FFA composition of BMSC-derived preadipocytes. **(F)** chromatogram of FFA composition of BMSC-derived adipocytes. **(G)** chromatogram of FFA composition of the culture medium. In **A**, the asterisk indicates statistical significance ($P < 0.05$) and refers only to intra-group statistical comparisons. In **B–E** the peaks labeled with asterisks are contamination compounds; for example, 1,1-dimethoxydecane, phthalate derivatives, etc. FFA, free fatty acid.

the content of stearic, oleic, linoleic, linolenic, and arachidonic acids. We showed that the initial FFA composition in BMSCs did not change significantly as cells differentiated toward adipocytes. The adipocytes differentiated in vitro from BMSCs tend to maintain the FFA composition of their mesenchymal stem cell progenitors rather than adopt the normal FFA composition of an adult adipocyte. The reduced amounts of linoleic and linolenic acid in the BMSCs may deprive differentiating adipocytes of some essential signaling pathways that could impair their normal functions; in turn, this might hamper their efficacy for transplantation in vivo. Since the differentiation medium contained only small amounts of palmitic, stearic, and oleic acids, the differentiating cells did not acquire a normal composition of FFAs, comparable to that of an adult adipocyte.

In this investigation we used a very sensitive technique to compare the FFA composition in BMSC-derived adipocytes during and after differentiation to that of adult adipocytes and adipose tissue. Future studies can use this technique to investigate comparatively other classes of lipids or whether changes in the cell culture media might improve the FFA composition of adipocytes differentiated in vitro from BMSCs.

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Author Disclosure Statement

No competing financial interests exist.

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