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Depot-specific and hypercaloric diet-induced effects on the osteoblast and adipocyte differentiation potential of adipose-derived stromal cells

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ABSTRACT

Adipose-derived stromal cells (ADSCs) can be differentiated *in vitro* into several mesenchyme-derived cell types. We had previously described depot-specific differences in the adipocyte differentiation of ADSCs, and consequently we hypothesized that there may also be depot-specific differences in osteoblast differentiation of ADSCs. For this study, the osteoblast differentiation potential of rat subcutaneous ADSCs (scADSCs) and perirenal visceral ADSCs (pvADSCs) was compared. Osteoblast differentiation media (OM) induced markers of the osteoblastic phenotype in scADSCs, but not in pvADSCs. ADSCs harvested from rats with diet-induced visceral obesity (DIO) exhibited reduced osteoinduction, compared to lean controls, but adipocyte differentiation was not affected. Expression of the pro-osteogenic transcription factor Msx2 was significantly higher in naïve scADSCs from lean and DIO rats than in pvADSCs. Our findings indicate that ADSCs from different anatomical sites are uniquely pre-programmed *in vivo* in a depot-specific manner, and that diet-induced metabolic disturbances translate into reduced osteoblast differentiation of ADSCs.

1. Introduction

Multipotential stromal cells reside in many adult tissues and can give rise to several mesenchyme-derived cell types, such as osteoblasts, adipocytes, myoblasts and chondrocytes (Song and Tuan, 2004). Bone marrow has classically been used as a source of stromal cells, especially for studying osteoblast formation *in vitro*. However, due to its abundance and ease of isolation, there is currently growing interest in the use of adipose tissue as an alternative source of stromal cells for both research and clinical applications (Fraser et al., 2006; Hodgkinson et al., 2009). These stromal cells can be harvested from either subcutaneous or visceral adipose tissue. As it is well established that there are fundamental metabolic differences between these two depots (Jensen, 2008), we have previously compared the capacity of rat adipose-derived stromal cells (ADSCs) from inguinal subcutaneous (scADSCs) and perirenal visceral fat (pvADSCs) to differentiate into functional adipocytes *in vitro* (Sadie-Van Gijsen et al., 2010), and found that pvADSCs exhibited a stronger adipogenic response than scADSCs. Given this difference in adipogenic potential between ADSCs isolated from different adipose depots, we hypothesized that stromal cells from visceral and subcutaneous adipose tissue may also exhibit differences in osteoblast differentiation potential.

Functional studies have demonstrated that ADSCs from human and rodent origin have the ability to differentiate into osteoblasts *in vitro* (Zuk et al., 2001; Huang et al., 2002; Yoshimura et al., 2007) and form bone tissue *in vivo* (Lee et al., 2003; Jeon et al., 2008). However, to our knowledge, no previously published studies have directly quantitatively compared the osteoblast differentiation potential of either rat or human ADSCs from different adipose sites. In addition, there is a paucity of data available regarding the effects of diet and metabolic status *in vivo* on the *in vitro* adipocyte and osteoblast differentiation potential of rat ADSCs from different adipose tissues. Diet-induced obesity and the resultant metabolic

Abbreviations: ALP, alkaline phosphatase; AM, adipocyte differentiation media; ARBP, acidic ribosomal phosphoprotein; ARS, Alizarin Red S; BSP, bone sialoprotein; OM, osteoblast differentiation media; ORO, Oil Red O; pvADSCS, perirenal visceral adipose-derived stromal cells; scADSCs, subcutaneous adipose-derived stromal cells.

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sequelae, such as insulin resistance and type II diabetes, have considerable impact on both bone and adipose tissue *in vivo* (De Paula et al., 2010; Hough, 1989). With growing interest in ADSCs as a source of progenitor cells for therapeutic applications (Hodgkinson et al., 2009) and increasing global prevalence of diet-induced obesity and the metabolic syndrome (Centers for Disease Control and Prevention, 2004), it was considered prudent to investigate the effects of the life-style and metabolic status of the ADSC donor on progenitor cell function and differentiation potential.

In the present study, we compared the osteogenic potential of rat ADSCs isolated from inguinal subcutaneous fat (scADSCs) and perirenal visceral fat (pvADSCs). Markers of osteoblast function (deposition of calcified matrix and increased alkaline phosphatase (ALP) activity), changes in proliferation and the expression of the osteoblast-associated transcription factors Runx2 (Ducy, 2000) and Msx2 (Satokata et al., 2000) were measured to assess osteoblast differentiation of ADSCs from the two depots. We also investigated the effect of obesity, as induced by excessive energy intake, on the *in vitro* adipocyte and osteoblast differentiation potential of ADSCs from subcutaneous and visceral adipose depots.

2. Materials and methods

2.1. Experimental animals and isolation of ADSCs

All experiments involving animals were performed in accordance with the South African Medical Research Council *Guidelines on Ethics for Medical Research* and were approved by the University of Stellenbosch Ethics Committee. This complies with the South African Animal Protection Act (Act No. 71 of 1962).

Adult male Wistar rats (200 g) were placed on either a highly palatable diet (65% of energy as carbohydrates, 19% as protein and 16% as fat) (Pickavance et al., 1999) or a standard lab-chow diet (60% of energy as carbohydrates, 30% as protein and 10% as fat) for 16 weeks (Du Toit et al., 2008). The palatable diet contained 33% condensed milk and 7% sucrose by weight, and was designed to induce excessive energy intake without major changes in macronutrient composition, compared to the standard lab-chow diet (Pickavance et al., 1999). We have previously described the effects of this diet on adult male rats (Du Toit et al., 2008), which included a modest increase in total body weight, coupled with a pronounced increase in visceral fat mass. For this reason, the rats on this diet were designated DIO (diet-induced obesity). DIO rats were also dyslipidaemic, with increased plasma triglycerides and decreased high-density lipoprotein (HDL) levels, as well as being hyperinsulinaemic (Du Toit et al., 2008). Circulating angiotensin II and tumour necrosis factor-alpha (TNF-α) levels were also significantly elevated in DIO rats, while fasting blood glucose levels and blood pressure in both lean control and DIO rats were within the normal range. The homeostasis model assessment (HOMA) values for DIO rats were significantly higher than for the lean control rats (12.5 ± 2.0 for DIO rats vs 6.2 ± 0.7 for lean rats; $P < 0.05$) (Du Toit et al., 2008), indicative of diet-induced insulin resistance. ADSCs were isolated from inguinal subcutaneous (scADSCs) and perirenal visceral (pvADSCs) adipose depots from lean and DIO rats by means of collagenase digestion (Huang et al., 2002), with modifications as described previously (Sadie-Van Gijsen et al., 2010). Isolated scADSCs and pvADSCs from lean control animals were designated lean subcutaneous and lean visceral (LS and LV cells), respectively, and those from DIO rats DIO subcutaneous and DIO visceral (DS and DV cells).

2.2. Cell culture

Cell cultures were maintained at 37 °C, in 95% humidified air with 5% CO_2. Standard cell culture media consisted of high-glucose Dulbecco's Modified Eagle Medium (DMEM, Sigma–Aldrich, Schnelldorf, Germany) with 1% Penicillin/Streptomycin (Lonza, Walkersville, MD, USA) and 10% fetal bovine serum (FBS, Invitrogen, Paisley, UK), heat-inactivated before use for 30 min at 56 °C. Cultures were maintained until near-confluence and subcultured as required. For culture expansion, cells were treated with 0.5% trypsin solution (Lonza) for 1 min and subcultured at a dilution between 1:3 and 1:10. All experimental procedures were performed on confluent cells at passage 2, unless stated otherwise.

2.3. Differentiation media

Components for both osteoblast differentiation media (OM) and adipocyte differentiation media (AM) were purchased from Sigma. OM consisted of standard culture media plus 10 nM dexamethasone, 10 mM glycerol-2-phosphate and 0.05 mM ascorbic acid (Jaiswal et al., 1997). Glycerol-2-phosphate and ascorbic acid were dissolved in DMEM, and ascorbic acid was prepared fresh before use. AM consisted of standard culture media plus 10 μM insulin, 56 μM indomethacin, 500 μM 3-isobutylmethylxanthine and 1 μM dexamethasone (Ogawa et al., 2004). Both OM and AM were made fresh before use and replaced every 2–3 days. For experimental controls, cells were treated with standard culture media containing 0.1% EtOH.

2.4. Measuring of alkaline phosphatase (ALP) activity

Cells were seeded into 100 mm culture dishes, maintained in standard culture media until confluent and subsequently treated as indicated in the figure legends. The method for ALP extraction was adapted from that of Merchant-Larios et al. (1985). All steps were performed at 4 °C. Briefly, cells were washed with PBS and lysed with 800 μl lysis solution (10 mM Tris, pH 7.4; 1% Triton X-100; 2 mM PMSF in isopropanol). Cell lysates were collected in microcentrifuge tubes and subjected to centrifugation at $15\,000 \times g$ for 15 min. Cleared supernatants were transferred to clean microcentrifuge tubes and 0.2 volumes of ice-cold butanol added. Samples were mixed thoroughly, incubated for 45 min on a rotary wheel and phase separation performed by centrifugation at $15\,000 \times g$ for 1 h. The aqueous lower phase was transferred into a clean centrifuge tube and 0.6 volumes of cold acetone added. Samples were mixed thoroughly and precipitates collected at $15\,000 \times g$ for 60 min. The precipitate was resuspended in 20 μl of lysis solution without PMSF and stored at −20 °C. For microwell assays, 10 μl extract was added to 170 μl assay buffer (100 mM glycine, 1 mM $MgCl_2$, 1 mM $ZnCl_2$, pH 10.4) and 20 μl para-nitrophenylphosphate (Amersham Life Sciences, Little Chalfont, UK; 60 mM in 1.5 mM $MgCl_2$, pH 5.4) (www.sigmaaldrich.com). Formation of the yellow product, para-nitrophenol, at 37 °C was measured spectrophotometrically every 2 min for 20 min at 405 nm. Extracts were diluted 1:1 with water and the protein content measured by the Bradford method (Bradford, 1976).

2.5. Alizarin Red S (ARS) mineral staining

For the staining of mineralized nodules with ARS (Reinholz et al., 2000), confluent cultures were treated with OM for 28 days. The media was then removed and cells washed twice with PBS. Cells were fixed in 70% EtOH for 5 min, rinsed twice with deionized water and incubated with ARS (Amresco, Solon, OH, USA: 40 mM in deionized water, pH 4.0–4.1 set with 5% NH_4OH) for 16 h at room temperature. The dye solution was removed by aspiration and rinsed as follows: three times with deionized water, once with PBS and another three times with deionized water. Stained cultures were visualized using light microscopy. For quantification of ARS staining, the calcium-bound stain was extracted with