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Secreted adiponectin as a marker to evaluate *in vitro* the adipogenic differentiation of human mesenchymal stromal cells

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Abstract

Background aims. Multipotency is one of the hallmarks of mesenchymal stromal cells (MSCs). Given the widespread adoption of MSC-based clinical applications, the need for rapid and reliable methods to estimate MSC multipotency is demanding. Adipogenic potential is commonly evaluated by staining cell lipid droplets with oil red O. This cytochemical assay is performed at the terminal stage of adipogenic induction (21–28 days) and necessitates the destruction of the specimen. In this study, we investigated whether it is possible to assess MSC adipogenic differentiation in a more efficient, timely and non-destructive manner, while monitoring *in vitro* secretion of adiponectin, a hormone specifically secreted by adipose tissue. *Methods.* A commercially available enzyme-linked immunosorbent assay kit was used to quantify adiponectin secreted in the culture medium of adipo-induced human bone marrow–derived MSCs. Oil red O staining was used as a reference method. *Results.* Adiponectin is detectable after 10 days of induction at a median concentration of 5.13 ng/mL. The secretion of adiponectin steadily increases as adipogenesis proceeds. Adiponectin is undetectable when adipogenic induction is pharmacologically blocked, inefficient or when human MSCs are induced to differentiate toward the osteogenic lineage, proving the specificity of the assay. Furthermore, the results of adiponectin secretion strongly correlate with oil red O quantification at the end of induction treatment. *Conclusions.* Our results demonstrate that quantification of secreted adiponectin can be used as a reliable and robust method to evaluate adipogenic potential without destroying samples. This method provides a useful tool for quality control in the laboratory and in clinical applications of human MSCs.

Key Words: *adipogenesis, adiponectin, mesenchymal stromal cells, quality control*

Introduction

Human mesenchymal stromal cells (hMSCs) are pluripotent cells that are maintained in limited number in several adult tissues, such as bone marrow and fat [1,2]. hMSCs are classically characterized by a panel of cluster of differentiation markers and by their ability to differentiate toward the osteogenic, adipogenic and chondrogenic lineage *in vitro*. Once differentiated, hMSCs display both morphological and functional characteristics of mature osteoblasts, adipocytes or chondrocytes in terms of activation of signaling pathways and secretion of specific proteins [3]. Multipotency renders hMSCs an interesting candidate for therapeutic applications that address the repair and the regeneration of damaged tissues. In fact, in the past decade, many clinical applications of hMSCs have been explored in which *ex vivo* expansion is often required [4–8]. Several investigators have described that hMSCs have a limited lifespan and that their differentiative capacity is impaired by long-term culture [9,10]. Therefore, it is important to ensure that the multi-potentiality of hMSCs is preserved during the entire expansion to obtain a defined and consistent final cell product. For this reason, it is fundamental to investigate new assays able to evaluate the differentiation potential of hMSCs in a rapid manner.

The differentiation toward the adipocyte lineage can be easily demonstrated by the distinctive morphology of adipocytes that store energy in the form of triglyceride-filled lipid droplets in the cytoplasm. Indeed, oil red O staining is the gold standard

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assay to evaluate the adipogenic differentiation of hMSCs *in vitro* because the dye enables the visualization of intracellular lipid droplets after 21–28 days of differentiation [11,12]. Several other methods are used to demonstrate hMSC adipo-potency, including the detection of protein or RNA expression of specific molecular markers such as the peroxisome proliferator-activated receptor γ (PPARγ), the CCATT/enhancer binding protein (C/EBP) [13] and the fatty acid–binding protein 4 (FABP4) [12,14,15]. All the mentioned methods are sample-destructive and do not allow the non-invasive assessment of the adipogenic differentiation. Especially for cases in which cells are cultured for clinical use, it is crucial to develop an alternative assay to test differentiation toward the adipocyte lineage while preserving the sample and delivering the results in a shorter time frame, with a significant impact on the cost of the production of the expanded cells.

Adipogenic differentiation in hMSCs is the result of a synergic interaction between different signaling pathways, including PI3K/Akt/mTOR or MAPK/ERK. In fact, specific inhibitors of key molecules of these pathways such as Wortmannin for AKT or LY294002 for PI3K have been used to block adipogenic differentiation [16,17].

Adiponectin [18,19], as well as leptin, adipsin, and resistin, is a hormone specifically produced by adipose tissue [20]. This protein is composed of 247 aminoacids and it is present in human blood as several isomers: trimeric, hexameric oligomers and high-molecular-weight multimers [21]. In the past decade, several researchers have directed their attention to a possible association between the serum levels of adiponectin and specific diseases or health conditions such as diabetes, osteoporosis, and functionality of epithelial progenitor cells [22–24] with the use of enzyme-linked immunosorbent assays (ELISA) available on the market.

Adiponectin expression, in terms of messenger RNA (mRNA) or intracellular protein level, has also been used by some authors as a marker of adipogenic differentiation of hMSCs [15,25–27]. In the present study we investigate the adiponectin secretion profile during *in vitro* adipo-induction of hMSCs with the aim to propose an alternative, sample-preserving method of evaluating adipogenic potential of hMSCs for clinical application.

Methods

Isolation and culture of hMSCs

hMSCs were obtained from the bone marrow of 13 subjects who underwent elective surgery at Rizzoli Orthopaedic Institute. Bone marrow mononucleated cells were isolated by means of gradient separation (Ficoll-Paque PREMIUM, GE Healthcare, Uppsala, Sweden; density, 1.073 g/ mL) and plastic adherence as previously described [28]. After the isolation of mononucleated cells, their viability was evaluated with the use of a NucleoCounter device (ChemoMetec A/S, Allerød, Denmark), and 400,000 cells/cm^2 were seeded in complete medium composed of α-modified minimum essential medium (α-MEM; BioWhittaker, Lonza, Verviers, Belgium) supplemented with 20% lot-selected fetal bovine serum (FBS; Lonza, Basel, Switzerland), 1% GlutaMAX (Gibco, Life Technologies, Paisley, United Kingdom). After 48 h of culture, the medium was changed to discard non-adherent cells. When cells reached 70–80% confluence, they were detached by trypsinization (TripLe Select; Life Technologies) for 5 min at 37°C, counted and expanded at a seeding density of 2000 cells/cm^2.

Cell lineage differentiation

For adipogenic induction, hMSCs were induced to differentiate toward the adipogenic lineage as previously described [29]. In brief, 5×10^5 cells were resuspended in 1.5 mL of Dulbecco's modified essential medium–high glucose (DMEM-HG; Euroclone, Milan, Italy) supplemented with 2% FBS (Gibco) and plated in each well of a six-well plate (Corning, Life Sciences, Amsterdam, the Netherlands). Three wells were maintained in control media and three wells were treated with adipogenic induction medium from the day after onwards. The adipogenic medium was composed of DMEM-HG supplemented with 2% FBS, 10 μmol/L bovine insulin (Sigma, St Louis, MO, USA), 1 μmol/L dexamethasone (Sigma), 200 μmol/L indomethacin (Sigma) and 500 μmol/L of 3-isobutyl-1-1methylxanthine (Sigma). hMSCs were induced for 21 days with a complete medium change, twice per week. Cells at passage P3-P5 were used and tested in triplicate for each of the 13 donors.

Oil red O staining and quantification

Adipogenic differentiation was evaluated with the use of oil red O staining. In brief, 21 days after adipogenic induction, or earlier when indicated, hMSC cultures were washed twice with phosphate-buffered saline (Euroclone, Milan, Italy), fixed in 4% paraformaldehyde (Sigma) for 10 min at room temperature and stained with a mixture of three volumes of oil red O (Sigma) 0.3% in 2-propanol and two volumes of deionized water for 15 min at room temperature. Differentiated cells were recognized by their round shape and presence of intracellular lipid droplets, and bright-field images of representative fields were acquired with a phase-contrast microscope (Nikon