Characterization Of Bovine Adipose-Derived Stem Cells

Daniel Cebo

ABSTRACT: Bovine adipose-derived stem cells were obtained from the subcutaneous abdominal adipose tissue. The cells were cultured by the modified tissue-explants method developed in our laboratory, and then analyzed using optical microscopy and flow cytometry. These cells were able to replicate in our cell culture conditions cell Flow cytometry showed that bovine adipose-derived stem cells expressed mesenchymal stem cell markers (CD73 and CD90). Meanwhile, haematopoietic markers (CD45 and CD34) are absent from bovine adipose-derived stem cells We also induced their adipogenic differentiation *in vitro*.

KEYWORDS: adipocytes, adult stem cells, mesenchymal stem cells, regenerative medicine

1. INTRODUCTION

Adipose tissue is an abundant source of mesenchymal stem cells, which have shown promise in the field of regenerative medicine [1]. There is a growing scientific interest in the plasticity and therapeutic potential of adipose-derived stem cells (ASCs). ASCs are multipotent, differentiating along the adipocyte, chondrocyte, myocyte, neuronal, and osteoblast lineages [2]. ASCs have potential applications for the repair and regeneration of acute and chronically damaged tissues [3]. While embryonic stem cells (ESCs) exhibit unlimited differentiation potential, the application of ESCs in cell-based therapies is limited by ethical and legal issues. Mesenchymal stem cells (MSCs) especially cells derived from adipose tissue also show great differentiation potential. ASCs have been used in studies of osteoarthritis, diabetes mellitus, heart disease, and soft tissue regeneration and reconstruction after mastectomy and facial repair [4]. Many kidney diseases are associated with inflammation and altered immune response. ASCs are known for their anti-inflammatory properties and immune modulation. Demonstration that the phenotype and immunosuppressive ability of ASCs are not affected by human kidney disease could have clinical significance [5, 6]. In order to be able to obtain ASCs cell preparations suitable for basic investigations as well as for development of future therapeutic protocols, it is important that the critical isolation steps are properly carried out. Here, we describe the isolation of ASCs using a tissue explants-based procedure from the bovine subcutaneous fat tissue that is also adaptable to several animal species. The aim of this study was to isolate, cultivate and identify adipose-derived stem cells.

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2. MATERIALS AND METHODS

2.1. Materials

Dexamethasone, 3-isobutyl-1-methylxanthine, insulin, Dulbecco's modified Eagle's medium (DMEM/F12), DMEM/Ham's nutrient mixture F12, Hanks' balanced salt solution (HBSS), and 100× antibiotic-antimycotic solution were all purchased from Sigma-Aldrich (St. Louis, MO). Foetal bovine serum was purchased from Atlanta Biological (Lawrenceville, GA). Trypsin-EDTA was purchased from Mediatech Inc. (Herndon, VA). Cell markers: fluorescein isothiocyanate (FITC), allophycocyanin (APC) or phycoerythrin (PE) are purchased from BD Biosciences, USA.

2.2. Animals

Adipose tissue was collected from beef cattle slaughtered at a local abattoir. Subcutaneous adipose tissue was transported to the laboratory in cold HBSS supplemented with 10% antibiotic-antimycotic solution. Tissues were collected post-mortem from animals slaughtered for food and not for research; therefore, Institutional Animal Care and Use Committee approval was not required

2.3. Culture and expansion

Tissue was extensively washed with phosphate buffered saline (PBS) to remove contaminating debris. After removing excess water, the samples were minced into very small pieces (less than 5 mm) with scissors and placed in tissue culture flasks under sterile conditions. The spacing between adjacent tissues was around 8-10 mm. Then the flask was tipped up on its side, with the cap loosened by 1/4 turn to allow CO_2 atmosphere exchange, and the tissue was let to "dry" at the bottom of the flask for 1-2 hours. The flask was gently laid back down, allowing media to again surround and cover the tissue. After the explants adhered to the bottom, the flask was gently laid back down, allowing growth medium containing D-MEM (without FCS) to surround and cover the tissue. The flasks were maintained in a humidified atmosphere of 5% CO_2 at 37 °C and the medium was changed every day. On the day 7, medium was changed to D-MEM with 20% FCS. Medium was changed every second day. On the day 14, medium was changed to D-MEM with 10% FCS. When the cells grew to 80% confluence they were passaged using standard trypsinization techniques cells were counted using the Trypan Blue exclusion test (haemocytometer). 1.0 x 10^6

cells were seeded in each new flask and the flasks were kept in humidified incubator (37 ºC, 95% O_2 and 5% CO_2).

2.4. Adipogenic differentiation

For adipogenesis, cells were seeded at a density of 2 x 10^4 cells/cm^2 and incubated in D-MEM with 10% FCS for 48 h to enable them to adhere to the plates. The medium (D-MEM without FCS) was changed every second day for 7 days. After 10 days the medium was then changed to adipogenic medium containing D-MEM, 10% FCS, 1 mM dexamethasone, 10 mM insulin, 200 mM indomethacin, and 0.5 mM isobutyl-methylxanthine. The medium was replaced every 2 days. After 1 week of induction the induced cells were analyzed.

2.5. Immunophenotypic characterization of adipose-derived stem cells

ASCs were tripsinized and the cellular suspension was centrifuged. ASCs were stained with antibodies conjugated with FITC, APC or PE: CD34-APC, CD45-FITC, CD73-PE, CD90-APC. The cells were resuspended in 0.3 ml PBS and incubated with FITC-, APC- or PE-conjugated antibodies for 35 minutes at room temperature and protected from light. The samples were analyzed by flow cytometry.

3. RESULTS

3.1. Differentiation assay

ASCs in this study were evaluated from first to last passage. Those cells showed morphology similar to fibroblasts (Figure 1). Cell morphology was modified during adipogenic differentiation presenting lipid droplets one week of stimulation with adipogenic medium showing big lipid inclusions inside the cytoplasm, indicating the adipogenic capacity of this cells which was confirmed by optical microscopy (Figure 2).

3.2. Cell surface markers

Cellular surface markers (CD) were utilized on second and third passage-cells to characterize the ASCs population. Flow cytometry results showed that these cells expressed CD73, CD90 accepted as mesenchymal stem cell markers but did not express haematopoietic cells markers (CD34 and CD45).

4. DISCUSSION

Adipose tissue stem cells can be harvested from adipose tissue and expanded, with capacity of differentiation on multiple lineages *in vitro* [7, 8]. Recently, many adipose tissue stem cells isolation methods have been developed which demonstrated that adipocytes can be isolated from the stromal vascular fraction using enzymatic digestion. It was showed later that this fraction contained a stem cell population [9]. But collagenase enzyme used in this procedure is an expensive reagent derived from a bacterial source, and its use in isolating ASCs is a time-consuming procedure. However, it hasn't yet been developed any efficient method to isolate and identify this cell population by explants-derived cell culture system. We showed in this paper a way to isolate this cell population using modified tissue-explants cultivation method with subsequent cell proliferation and efficient potential of *in vitro* differentiation. Mesenchymal stem cells do not have a specific marker, which make difficulties in the identification of these cells by specific antibodies. Instead, multiple markers are used together to identify this tissue. In this study, we selected the following markers for the adipose tissue stem-cells identification: CD73, CD90 (mesenchymal stem cell markers) and CD34 and CD45 (haematopoietic cell markers). Fat tissue fragments attached to the flasks showed some fibroblast-like morphology on days 10-14 in the presence D-MEM with FCS and on the days 15 the cells began proliferate rapidly and when the cells grew to 80% confluence they were passaged using standard trypsinization techniques. The cells were also analyzed by flow cytometry (Table 1). The flow cytometry analysis showed that from the second passage cells were positive for CD73-PE, CD90-APC with expression rates up to 90%, but negative for CD34-APC and CD45-FITC, with a 3% rate. The cells maintained their phenotypes throughout the studied period on different passages, a fact that confirmed their suitability for *in vitro* cultivation. We also induced their adipogenic differentiation, verifying their mesenchymal stem cells potentiality *in vitro*. The morphological aspect of young cells with fusiform shape is a characteristic of ASCs where multilocular lipid droplets can be identified in the cytoplasm due to the fat conversion occurring in these cells. Such adipose cell characteristic, before forming a unique lipid inclusion, illustrates the adipocytes differentiation step.

5. CONCLUSIONS

The adipose tissue stem cells can be efficiently isolated using the described methodology, kept similar morphology to fibroblasts when cultivated in the evaluated period and demonstrated adipogenic, osteogenic and chondrogenic differentiation potential *in vitro*. Our proposed method for cell culture following cell separation by tissue-explants based method may provide the opportunity to characterize other adipogenic or anti-adipogenic factors related to adipose tissue expansion control. Of special interest is the fact that it was possible to isolate precursor cells of bovine adipose tissue, by *in vitro* cultivation, maintaining their functional integrity. Finally, we believe that the proposed model may add new knowledge about the lipid droplets biology, contributing to studies involving tissue engineering with quality and reproducibility.

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CONFLICT OF INTEREST STATEMENT

This paper does not contain any conflict of interest.

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Figure 1. The cell morphology of adipose-derived stem cells (ASCs) *A) The cells showed a long spindle-like shape 7 days after fibroblastic differentiation. B) Fourteen days after fibroblastic differentiation, the ASCs were more homogeneously fibroblast-like and typically spindle shaped, with a fascicular arrangement.*

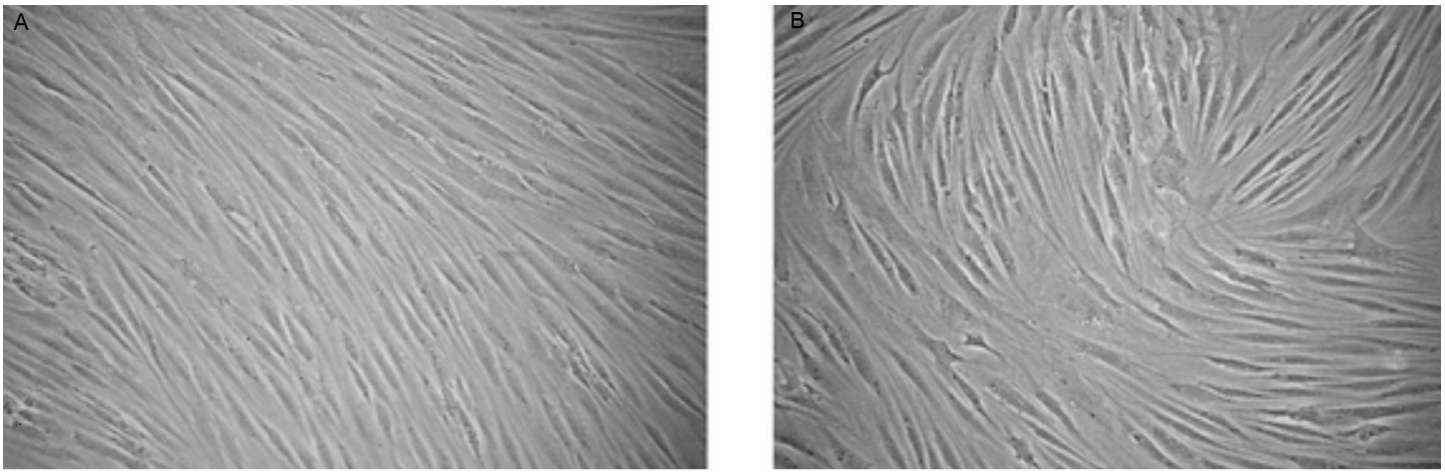

Figure 2. The cell morphology of the cells after one week of stimulation with radiogenic medium. *Cells shows adipocytes morphology with big lipid droplets inside cytoplasm.*

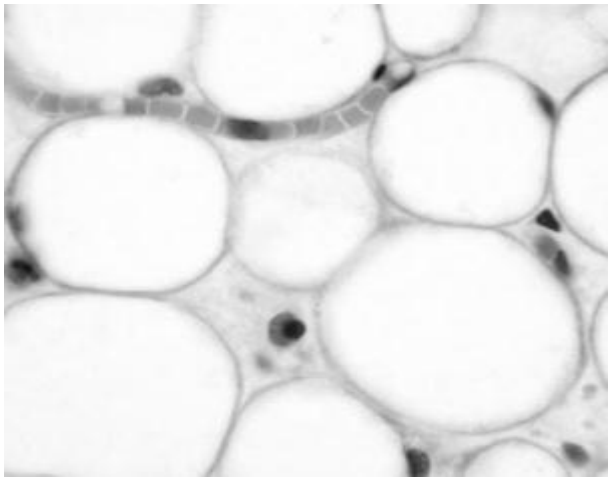

Table 1. Flow cytometry analysis*. Cells from the second passage were positive for CD73-PE and CD90-APC with expression rates up to 95%, but negative for CD34-APC and CD45-FITC.*

2. Passage	3. Passage
95% CD73-PE/CD90-APC 3% CD34-APC/CD45-FITC	96-98% CD73-PE/CD90-APC 2-3% CD34-APC/CD45-FITC