Characterization of human adipose-derived stem cells[1]

Caracterização de células-tronco do tecido adiposo humano

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ABSTRACT

PURPOSE: There is a growing scientific interest in the plasticity and therapeutic potential of adipose-derived stem cells (ASCs), which are multipotent and abundant in adipose tissue and can differentiate *in vitro* into multiple lineages, including adipocytes, chondrocytes, osteoblasts, neural cells, endothelial cells and cardiomyocytes. The aim of this study was to isolate, cultivate and identify ASCs.

METHODS: Human adipose precursor cells were obtained from subcutaneous abdominal tissue. Recently dispersed cells were separated by density centrifugation gradient, cultured and then analyzed.

RESULTS: Human ASCs were able to replicate in our culture conditions. The cells maintained their phenotypes throughout the studied period on different passages confirming they suitability for *in vitro* cultivation. We also induced their adipogenic, osteogenic and chondrogenic differentiation, verifying their mesenchymal stem cells potentiality *in vitro*. Flow cytometry results showed that these cells expressed CD73, CD90 and CD105, (mesenchymal stem-cells markers), contrasting with the lack of expression of CD16, CD34 and CD45 (hematopoietic cells markers).

CONCLUSION: It was possible to isolate human adipose-derived stem cells by *in vitro* cultivation without adipogenic induction, maintaining their functional integrity and high proliferation levels. The cells demonstrated adipogenic, osteogenic and chondrogenic differentiation potential *in vitro*.

Key words: Adipocytes. Adult Stem Cells. Mesenchymal Stem Cells. Stem Cells. Adipose Tissue.

RESUMO

OBJETIVO: Há um interesse científico crescente na plasticidade e potencial terapêutico das células-tronco do tecido adiposo humano, células multipotentes e abundantes no tecido adiposo que podem se diferenciar *in vitro* em múltiplas linhagens celulares, incluindo adipócitos, condrócitos, osteoblastos, células neurais, endoteliais e cardiomiócitos. O objetivo deste estudo foi isolar, cultivar e identificar células-tronco do tecido adiposo humano.

MÉTODOS: Células precursoras humanas do tecido adiposo foram obtidas de tecido abdominal subcutâneo. As células recém-dispersas foram separadas por gradiente de centrifugação por densidade, cultivadas e então analisadas.

RESULTADOS: As células-tronco do tecido adiposo humano foram capazes de se replicar nas nossas condições de cultivo e mantiveram seu fenótipo em diferentes passagens durante o estudo, confirmando sua adequabilidade para cultivo *in vitro*. A diferenciação adipogênica, osteogênica e condrogênica também foi induzida, confirmando seu potencial de células-tronco mesenquimais *in vitro*. Os resultados de citometria de fluxo evidenciaram a expressão dos marcadores de células-tronco mesenquimais CD73, CD90 e CD105, contrastando com a falta de expressão dos marcadores de células hematopoiéticas CD16, CD34 e CD45.

CONCLUSÃO: Foi possível isolar células-tronco do tecido adiposo humano por cultivo *in vitro* sem indução adipogênica, mantendo sua integridade funcional e altos níveis de proliferação. As células demonstraram potencial de diferenciação adipogênico, osteogênico e condrogênico *in vitro*.

Descritores: Adipócitos. Células-Tronco Adultas. Células-Tronco Mesenquimais. Células-Tronco. Tecido Adiposo.

Introduction

Tissue reconstruction in patients with loss of substance of mesenchymal origin like fat, bone and muscle due to trauma, tumor resection or vascular damage, represents a clinical problem of difficult solution. In these cases, the use of autologous tissue to reconstruct soft parts is of great importance[1-6].

Two cell types are generally considered for this purpose: progenitor cells and stem cells. These cells present some limitations such as their diminished cell proliferation potential as the donor gets older and the low number of cells that can be collected[7-10]. On the other hand, the therapeutic potential of adult stem cells is enormous due to their self-renewal ability and multipotentiality. Adult stem cells are immunocompatible due to their autologous nature and their use does not involve ethical issues[11-15]. Bone marrow mesenchymal stem cells can be representative of this type due to their adipogenic, osteogenic, chondrogenic, myogenic and neurogenic potential *in vitro*[16-20]. There is a growing scientific interest in the plasticity and therapeutic potential of Human Adipose-Derived Stem Cells (ASCs). ASCs are multipotent and abundant in adipose tissue and they can differentiate *in vitro* into multiple lineages, including adipocytes, chondrocytes, osteoblasts, neural cells, endothelial cells and cardiomyocytes[21].

The adipose tissue is the body's major energy reserve, being almost ubiquitously distributed over the body and it shows an enormous plasticity throughout life. The main cellular components are mature, lipid-filled adipocytes, lipid-free preadipocytes and endothelial cells. Adipocytes are the only cells specialized in storing lipid in their cytoplasm as triacylglycerol without affecting their functional integrity[22]. When the energy balance is positive, extra calories are stored as triacylglycerol, which is achieved by enlargement of adipocytes, as well as via recruitment of committed progenitor cells to form more adipocytes[23-24].

Correction of soft tissues by autologous fat graft is a common procedure in plastic surgery. Its efficacy and safety were considerably discussed and several lipoinjection techniques were developed[25]. However, this procedure must often be repeated due to resorption of the graft, which may occur because of the lack of nutritional support to the inner cell layers and because many of the injected cells are already differentiated[26-27].

Autologous adipose cells are cultured so that only non-differentiated, but committed, preadipocytes are grafted and this procedure can be done in a way that also ensures optimal nutritional support for the cells[26-27].

The aim of this study was to isolate, cultivate and identify adipose-derived stem cells (ASCs).

Methods

Isolation of human adipose-derived stem cells (ASCs)

Adipose tissue samples were obtained from the subcutaneous abdominal tissue of ten adult female patients, aged 32-49 years (mean=40 years old), submitted to abdominoplasty. Patients with inflammatory or malignant diseases were discarded. This research study was approved by the Research Ethics Committee of the Federal University of Sao Paulo and all patients signed a written free and informed consent form.

Cell isolation was done according to the method described by Hauner *et al*.[28] with modifications. Briefly, after removal of all fibrous material and visible blood vessels, adipose tissue samples were cut into small pieces (~10 to 20 mm) and digested in 10 mM Phosphate- Buffered Saline (PBS) (Sigma Aldrich, St. Louis, Missouri, USA) containing 200 U/ml crude collagenase II (Sigma Aldrich, St. Louis, Missouri, USA) for 40 minutes in a shaking water bath at 37ºC. The dispersed material was filtered through a nylon mesh with a pore size of 150 μm. After centrifugation (300g), the mature adipocytes remained on the supernatant surface and the ASCs formed a pellet at the bottom of the tube. Ficoll-hypaque (Histopaque®) was used as a centrifugation gradient for the separation of ASCs and the solution containing the pellet was resuspended in 3 ml of PBS. This solution was added to equal volumes of Histopaque® of different densities (1077 and 1119). The obtained mix was centrifuged (300*g*) for 30 minutes. Red blood cells (at the bottom of the tube) were, then, easily eliminated and the cells from the intermediate ring of the density gradient tube were used for both cytometric analyses and cultivation.

Culture and expansion

Cells were cultured in a standard medium composed by Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F12) (Sigma Aldrich, St. Louis, Missouri, USA), supplemented with 10% Fetal Bovine Serum (FBS) (Cultilab, Campinas-SP, Brazil), 100 U/ml penicillin (Sigma Aldrich, St. Louis, Missouri, USA) and 0.1 mg/ml streptomycin (Sigma Aldrich, St. Louis, Missouri, USA). The culture was maintained at 37ºC in humidified atmosphere of 95% O_2 and 5% CO_2. Cells were cultured for up to 6 passages, in triplicates. In each passage, $1x10^6$ cells were seeded to subconfluence in 75cm^2 culture flasks for 7-10 days.

When the cells attached to the flask reached approximately 80% confluence, subculture (passage) was performed through enzymatic digestion (tripsinization). Cells were used from the second to the sixth passage. For the tripsinization the culture

medium was aspirated and the flask was washed using a PBS solution supplemented with ethylenediamine-tetra acetic acid (EDTA) (0.5M). The solution was then discarded and 3 ml of a solution composed by tripsin (0.25%) plus EDTA (0.02%) was added per flask and left for two minutes. The tripsin solution was neutralized using 3 ml complete DMEM/F12 medium. The cell suspension was centrifuged (300g, ten minutes) and its supernatant was aspirated. The pellet was resuspended in complete DMEM/F12 medium and cells were counted using the Trypan Blue exclusion test (hemocytometer). 1.0 x 10^6 cells were seeded in each new flask and the flasks were kept in humidified incubator (37ºC, 95% O_2 and 5% CO_2).

Differentiation assays

Adipogenic differentiation

For the adipogenic differentiation, cells were cultivated in 6-wells plates (TPP, Trasadingen, Schaffhausen, Switzerland) containing 2 ml of complete DMEM/F12 medium supplemented with 10 µM insulin and 1 µM dexamethasone (Sigma Aldrich, St. Louis, Missouri, USA) for 15 days (adipogenic medium). The plates were kept in humidified incubator (37ºC, 95% O_2 and 5% CO_2) and the culture media was changed every three days. After this period, the medium was aspirated and the cells were washed twice with PBS. Then, 2 ml paraformaldehyde (Electron Microscopy Sciences) 0.4% in PBS was added to the cells. The fixative solution was aspirated after 30 minutes and the cells were washed three times with PBS as following: once with PBS containing Glycine 0.1 M for 10 minutes and twice with PBS for two minutes.

The cells were then incubated with Oil Red O dye (0.5%) at room temperature for 30 minutes. The dye was carefully removed with a 2 ml disposable pipette and the plate was washed five times with 2 ml water. The fixed and dyed cells were observed using Nikon Ti-U optical microscope and photographed using NIS-Elements - 3.2 Software (Nikon Instruments INC, New York).

Osteogenic differentiation

For the osteogenic differentiation, the cells were cultured in six wells plates (TPP, Trasadingen, Schaffhausen, Switzerland) containing 2 ml complete DMEM/F12 medium supplemented with 50µM ascorbic acid (Sigma Aldrich, St. Louis, Missouri, USA), 0.1µM dexamethasone and 10^{-2}M β glycerophosphate (Mallinckrodt Baker, Phillipsburg, New Jersey, USA) for 21 days (osteogenic medium). The plates were kept in humidified incubator (37ºC, 95% O_2 and 5% CO_2) and the culture media was changed every three days. After this period, the medium was aspirated and the cells were washed twice with PBS. Then, a solution of 2 ml paraformaldehyde (Electron Microscopy Sciences) 0.4% in PBS was added to the cells. The fixative solution was aspirated after 30 minutes and the cells were washed three times with PBS as following: once with PBS containing Glycine 0.1 M for ten minutes and twice with PBS for two minutes.

The cells were then incubated in a solution of sodium alizarin (40nM, pH 4.1) (Sigma Aldrich, St. Louis, Missouri, USA) at room temperature for 30 minutes. The dye was carefully removed with a 2 ml disposable pipette and the plate was washed five times with 2 ml water. The fixed and dyed cells were observed using Nikon Ti-U optical microscope and photographed using the NIS-Elements - 3.2 Software (Nikon Instruments INC, New York).

Chondrogenic differentiation

For chondrogenic differentiation, the cells were cultured in six wells plates (TPP, Trasadingen, Schaffhausen, Switzerland) containing 2 ml complete DMEM/F12 supplemented with 10 µM insulin, 0.1 µM dexamethasone, 50µM ascorbic acid and 10ng/ml TGF-β1 (Cell Signaling Technology, Beverly, Massachusetts, USA) for 21 days (chondrogenic medium). The plates were kept in humidified incubator (37ºC, 95% O_2 and 5% CO_2) and the culture media was changed every three days. After this period, the medium was aspirated and the cells were washed twice with PBS. Then the solution of 2 ml paraformaldehyde (Electron Microscopy Sciences) 0.4% in PBS was added to the cells. The fixative solution was aspirated after 30 minutes and the cells were washed three times with PBS as following: once with PBS containing Glycine 0.1 M for ten minutes and twice with PBS for two minutes.

The cells were then incubated in a Toluidine Blue solution (0.1%) (Sigma Aldrich, St. Louis, Missouri, USA) at room temperature for 30 minutes. The dye was carefully removed with a 2 ml disposable pipette and the plate was washed five times with 2 ml of water. The fixed and dyed cells were observed using Nikon Ti-U optical microscope and photographed using the NIS-Elements - 3.2 Software (Nikon Instruments INC, New York).

Immunophenotypic characterization of ASCs

ASCs from the second passage were used for the immunophenotypic characterization. The cells were tripsinized and the cellular suspension was centrifuged (300*g*, 4 min). ASCs were stained with antibodies conjugated with fluorescein isothiocyanate (FITC), allophycocyanin (APC) or phycoerythrin (PE): CD16-PE, CD34-APC, CD45-FITC, CD73-PE, CD90-APC and CD105-PE (BD Biosciences, USA). A total of 5 x 10^5 cells were resuspended in 0.2 ml PBS and incubated with FITC-, APC- or PE-conjugated

antibodies for 20 minutes at room temperature and protected from light. The samples were analyzed by flow cytometry (Guava easyCyte, Millipore) for identification of specific fluorescence channels of each antibody.

Results

ACSs differentiation assays

ASCs were isolated using enzymatic digestion with collagenase. The cultivated cells of this study were evaluated from first to sixth passage and all cells showed morphology similar to fibroblasts with fusiform characteristics (Figure 1A).

Cell morphology was modified during adipogenic differentiation presenting lipid droplets on the cytoplasm after the first week of culture and stimulation with adipogenic medium. After two weeks of stimulation, big lipid inclusions were found in the cytoplasm, indicating the adipogenic capacity of this cells (Figure 1B), which was confirmed by Oil red staining.

The ASCs cultivated in osteogenic medium showed morphology similar to osteoblasts after 21 days of stimulation, with the presence of calcium precipitates, confirmed by alizarin red staining (Figure 1C).

When the ASCs were cultivated in chondrogenic medium during 21 days, the cells showed similar morphology to chondrocytes, evidenced by Toluidine Blue staining (Figure 1D).

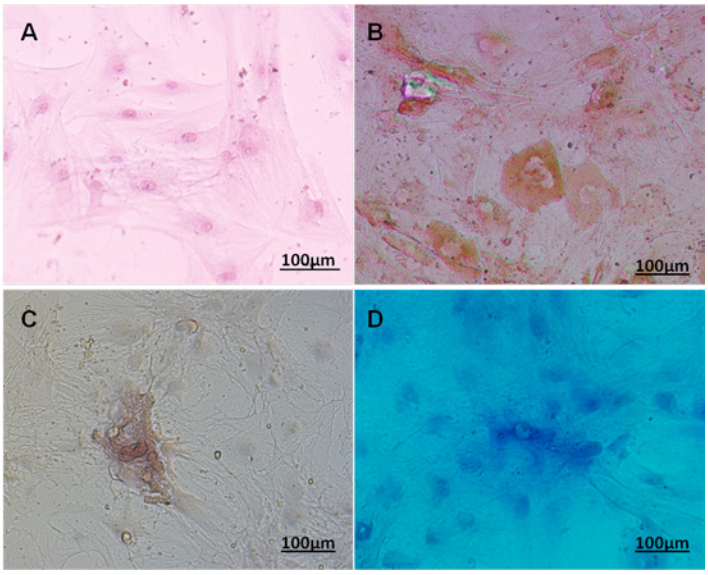

FIGURE 1 - (**A**) ASCs are typical fibroblast-like cells with fusiform shape. Here they were obtained at the second passage (p2). (**B**) ASCs - adipogenic differentiation. Lipids detected by Oil Red O staining after two weeks induction. (**C**) ASCs - osteogenic differentiation. Calcium nodule formation demonstrated by Alizarin red stain after three weeks induction. (**D**) ASCs - chondrogenic differentiation demonstrated by Toluidin blue stain after three weeks induction.

Cell surface markers

Cellular surface markers (CD) were utilized on second passage-cells to characterize the ASCs population. Flow cytometry results showed that these cells expressed CD73, CD90 and CD105, accepted as mesenchymal stem-cells markers, contrasting with the lack of expression of hematopoietic cells markers CD16, CD34 and CD45 (Figure 2).

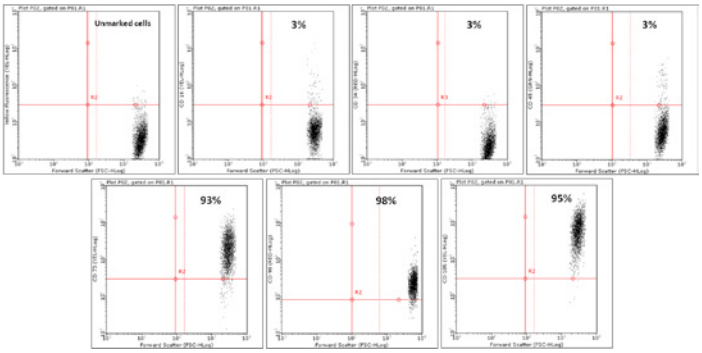

FIGURE 2 - Flow cytometry analysis. Cells from the second passage (P2) were positive for CD73-PE, CD90-APC, CD105-PE, with expression rates up to 95%, but negative for CD16-PE, CD34-APC and CD45-FITC.

Discussion

Adipose tissue stem cells can be easily harvested from adipose tissue and expanded, with capacity of differentiation on multiple lineages *in vitro*[21]. Many adipose tissue stem cells isolation methods have been developed since Rodbell[28], who demonstrated that adipocytes can be isolated from the stromal vascular fraction. It was showed later that this fraction contained a stem-cells population. However, it hasn't yet been developed any efficient method to identify this cell population[29]. We showed in this paper a way to isolate this cell population using enzymatic digestion with collagenase and centrifugation gradient separation (fycoll) with subsequent cell proliferation and efficient potential of *in vitro* differentiation.

Mesenchymal stem cells do not have a specific marker, which invalidates the identification of these cells by specific antibodies. Instead, multiple markers are used together to identify this tissue[21]. In this study, we selected the following markers for the adipose tissue stem-cells identification: CD73, 90, 105 (mesenchymal stem-cells markers) and CD16, 34 and 45 (hematopoietic cells markers).

After the separation process, and at the 2nd passage of cell culture, the cells were also analyzed by flow cytometry (Figure 2). According to size (FSC) and complexity (SSC) parameters, only one population of cells was observed, in the ASCs region (figure 2A), and flow cytometry analysis showed that the 2nd passage

cells were positive for CD73-PE, CD90-APC, CD105-PE, with expression rates up to 95%, but negative for CD16-PE, 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, osteogenic and chondrogenic differentiation, verifying their mesenchymal stem cells potentiality *in vitro*.

The morphological aspect of young cells with fusiform shape is a characteristic of ASCs (Figure 1) where multilocular lipid droplets can be identified in the cytoplasm due to the fat conversion occurring in these cells[14-16]. Such adipose cell characteristic, before forming a unique lipid inclusion, illustrates the adipocytes differentiation steps[29].

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 density centrifugation gradient 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 human 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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Acknowledgments

Special thanks to Raquel Cunkis Chiochette and Vanina Monique Tucci-Viegas for their generous help with the translation and revision of this manuscript.

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Received: February 08, 2012
Review: April 10, 2012
Accepted: May 14, 2012
Conflict of interest: none
Financial source: none

[1]Research performed at Skin Cell Culture Laboratory, Plastic Surgery Division, Department of Surgery, Federal University of Sao Paulo (UNIFESP), Brazil. Part of PhD thesis, Pastic Surgery Postgraduate Program Tutor: Lydia Masako Ferreira.