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Human mesenchymal stromal cells preserve their stem features better when cultured in the Dulbecco's modified Eagle medium

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Abstract

Background aims. The human mesenchymal stromal cell (hMSC), a type of adult stem cell with a fibroblast-like appearance, has the potential to differentiate along the mesenchymal lineage and also along other cell lineages. These abilities make hMSC a promising candidate for use in regenerative medicine. As the hMSC represents a very rare population *in vivo*, *in vitro* expansion is necessary for any clinical use. hMSC characterization is commonly carried out through the expression of specific markers and by the capability of differentiating toward at least adipo-, osteo- and chondrocytic lineages. Commitment processes also result in significant changes in the ultrastructure in order to acquire new functional abilities; however, few studies have dealt with the ultrastructural characteristics of hMSC according to the time of incubation and type of media. *Methods*. The immunophenotype, functional characteristics and ultrastructural features of bone marrow (BM) hMSC cultured in two different media were investigated. The media chosen were Iscove's modified Dulbecco's medium (IMDM) and the Dulbecco's modified Eagle medium (DMEM). The latter has been recommended recently by two international transplantation and cytotherapy societies, the International Society of Cellular Therapy (ISCT) and European Group for Blood and Bone Marrow Transplantation (EBMT), for hMSC expansion for clinical applications. *Results and Conclusions.* The present results indicate that culture conditions greatly influence hMSC ultrastructural features, proliferation, growth and differentiation. In particular, our findings demonstrate that DMEM preserves the hMSC stem features better. Furthermore, the results obtained in IMDM suggest that a small size does not always correlate with conditions of cell immaturity and a greater proliferative potential.

Key Words: *bone marrow, Dulbecco's modified Eagle medium, electron microscopy, human mesenchymal stromal (stem) cells, Iscove's modified Dulbecco's medium, regenerative medicine*

Introduction

The last decade has seen a progressive growth of interest in the human mesenchymal stromal cell (hMSC), a type of adult stem cell with a fibroblast-like appearance, easily isolated by plastic adherence (1) from a variety of sources such as adipose tissue, synovial tissue, dental pulp and, most often, bone marrow (BM). hMSC have the potential to differentiate along mesenchymal cell lineages such as bone, fat, cartilage and tendon, both *in vitro* and *in vivo* (2,3). In addition, in the last few years, several works have reported a wider range of *in vitro* differentiative potentials for these cells (4–6). The capacity to differentiate into a variety of specialized cell types and the demonstration of their potential to migrate to injury sites with repair function, make hMSC promising candidates for use in regenerative medicine (7). Moreover, it is well documented that the hMSC show immunosuppressive properties *in vitro*, while contrasting results come from *in vivo* pre-clinical and clinical studies (8–10).

hMSC identification is commonly established on the basis of morphology, differentiative capacity to mesenchymal lineages and expression of a panel of cell-surface molecules (none exclusive) (11). Flow cytometric analysis has also shown that hMSC comprises a heterogeneous cell population including at least two subpopulations, small, rapidly self-renewing cells (RS-hMSC) and larger, slowly renewing or mature cells (SR-hMSC or m-hMSC), that can be distinguished by differences in differentiative potential, expression levels of surface epitopes and *in vivo* engraftment in

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non-hematopoietic tissues (12,13). In addition, among the RS-hMSC it is possible to identify agranular cells (RS-1) and granular cells (RS-2) (14). The authors suggest that the RS-1 pool generates the RS-2 cells during the lag phase, and the RS-2 pool gives rise to m-hMSC during the log phase of *in vitro* expansion.

Many studies have investigated hMSC morphologic, functional and molecular aspects in order to understand the stem characteristics and differentiation processes of these cells. Comparisons of data from different laboratories often show contradictions, depending on the use of diverse isolation protocols and different culture conditions (15,16), that might have implications regarding the composition of the selected subpopulations (17), and it has been reported that different types of culture media have serious impacts on the gene expression and proteome of hMSC (18).

As hMSC represent a very rare population *in vivo*, *in vitro* expansion is necessary for clinical use. Therefore, establishing both optimal culture conditions and common standards for the expansion of hMSC seems necessary for research advancement and the use of hMSC in clinical medicine.

hMSC identity, as well as acquisition of differentiative features after induction, are commonly established on the basis of expression of specific markers and, eventually, in changes in cell size. Few papers have dealt with the ultrastructural characteristics of hMSC (15,19-22), the majority of them investigating the changes inducted by differentiation toward different cell linages (19-21).

We investigated the immunophenotype, functional characteristics and ultrastructural features of BM hMSC cultured in Iscove's modified Dulbecco's medium (IMDM) and Dulbecco's modified Eagle medium (DMEM), in order to describe the effects of these two media on the morphology, proliferation and differentiative potential of the cells. We chose these two medium because the former, IMDM, was previously utilized for the expansion of BM-derived hMSC (11), while the latter, DMEM, is now strongly recommended by the International Society of Cellular Therapy (ISCT) and European Group for Blood and Bone Marrow Transplantation (EBMT) for hMSC expansion for clinical applications. The cells were studied at very early passages (P0/P1) to reduce vacuolization and other similar unspecific morphologic aspects generally occurring in culture (15).

Methods

Isolation and culture of hMSC

hMSC were obtained from BM aspirates of four patients, not affected by congenital pathologies, undergoing orthopedic surgery. Informed consent was obtained from all donors and all procedures were approved by the institutional ethical committee. Whole BM was collected in acid citrate dextrose-A (ACD-A) and centrifuged for 10 min at 700 ***g***; the layer of white blood cells (buffy coat) was recovered and total nucleated cells (TNC) were plated in 75-cm^2 flasks (1.6×10^5 TNC/cm^2) under two conditions: (a) IMDM, with L-glutamine and N2-hydroxyethylpiperazine-N2ethane sulfonic acid (HEPES) 25 mM (Euro-Clone, Milan, Italy), 50 μg/mL gentamycin (Schering-Plough SpA, Milan, Italy), 10% fetal bovine serum (FBS; HyClone, South Logan, UT, USA) and 2% Ultroser® G (UG; Pall BioSepra SA, Cergy-Saint-Cristophe, France) [complete (c) IMDM]; and (b) DMEM-low glucose (Gibco-Invitrogen, Milan, Italy) supplemented with 10% FBS (HyClone) and 1% antibiotic–antimycotic solution (cDMEM). Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. When the cultures were near confluence, the cells were removed from the flask by using 0.05% trypsin–ethylene diamine tetra acetic acid (EDTA) 0.02% mM and replated at a density of 10^4 cells/cm^2 (Pn + 1).

Colony-forming units–fibroblast frequency

Using the method of Castro-Malaspina with slight modifications, we measured the frequency of colony-forming unit–fibroblasts (CFU-F) as a surrogate marker for hMSC frequency of the sample (23): two 100-mm dishes were seeded with 5×10^5 TNC (1:10 diluted) from the BM collection. After incubation for 14 days at 37°C in 5% CO_2 and a fully humidified atmosphere, the cells were washed with Hanks' balance salt solution (HBSS), fixed with methanol and stained with Giemsa: visible colonies with 50 or more cells (the conventional value for defining a colony) (24) were counted and referred to 10^6 plated cells (number of CFU-F/10^6 TNC).

Immunophenotyping

At the first passage, hMSC were analyzed for expression of a number of cell-surface molecules using flow cytometry procedures; hMSC, recovered from flasks by trypsin–EDTA treatment and washed in HBSS and FBS 10%, were resuspended in flow cytometry buffer consisting of CellWASH (0.1% sodium azide in phosphate-buffered saline (PBS); Becton Dickinson, Milan, Italy) with 2% FBS. Aliquots (1.5×10^5 cells/100 L) were incubated with the following conjugated monoclonal antibodies: CD34–phycoerythrin (PE), CD45–fluorescein isothiocyanate (FITC), CD14–PE (in order to quantify hemopoietic–monocytic contamination), CD29–PE, CD44–FITC, CD166–PE, CD90–PE, CD73–PE, HLA-DP-Q-R–FITC, HLA-ABC–FITC, CD71–FITC (all from BD Pharmingen, San Diego, CA, USA), CD105–PE (Ancell, Bayport, MI, USA)