Liveyon Product Report

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TITLE: MSCs Derived from PURE® Product

Key terms: MSC, regenerative medicine, umbilical cord blood, cell therapy, stem cells

Abstract

MSCs (mesenchymal stem cells) can be isolated from Liveyon's **PURE®** products. These cells have characteristic MSC cell surface markers and can differentiate into bone, fat and cartilage.

Introduction

Multipotent mesenchymal stromal cells (MSCs) are of great interest for regenerative medicine. These cells secrete growth factors, cytokines and exosomes. MSCs are immune-privileged (meaning they can be donated between people without eliciting an inflammatory immune response). MSCs modulate the immune system by affecting T and B cell proliferation, dendritic cell maturation and natural killer cell activity[1]. In vitro, MSCs can differentiate into a variety of cell types; in animal studies, MSCs engraft into multiple tissues[2]. They have a twenty-year history in clinical trials, including numerous long-term studies that demonstrated safety[3,4]. MSCs can enable regeneration of damaged and diseased tissues through a number of different mechanisms and have a good record of safety.

MSCs (also known as "mesenchymal stem cells" or "medicinal signaling cells") can be derived from a variety of tissues. These include bone marrow, umbilical cord blood, umbilical cord tissues, adipose tissue, synovial and amniotic fluids. The International Society for Cellular Therapy (ISCT) proposed a set of standards to define human MSCs for both laboratory-based scientific investigations and for preclinical studies[5]. The MSC-defining criteria are: 1) plastic-adherence in cell culture conditions, 2) positivity for CD73, CD90, and CD105 while lacking expression of HLA-DR, CD45, CD34, CD14 or CD11b, and CD79α or CD19, and 3) ability to differentiate into adipocytes, chondrocytes and osteoblasts in vitro.

While MSCs from all tissue sources share these common characteristics, there are subtle and potentially important differences. MSCs from umbilical cord blood (CB) showed higher proliferation capacity[6]. CB-MSCs may have better anti-inflammatory activity, lower expression of senescence markers[7], and better karyotype stability[8] compared to marrow and adipose MSCs. In addition, isolation of adult-derived MSCs require a surgical procedure which may be painful for the patient. Since CB-derived MSCs may have greater accessibility and clinical value, it is important to define conditions where these cells can be readily isolated.

This paper outlines culture conditions where MSCs are isolated from cord blood cells, as present in the Liveyon **PURE®** product. These cells are highly proliferative, express the characteristic MSC cell surface markers and can be differentiated into multiple lineages.

Methods

A basal medium (ATCC) was supplemented to 5 ng/ml FGFb, 15 ng/ml IGF-1, 2.4 mM L-Alanyl-L-Glutamine and 7% FBS (prescreened to support the undifferentiated growth of MSCs). Liveyon **PURE PRO®** vials were thawed and transferred to a 50 ml centrifuge tube, using an 18 G needle and 3 ml syringe. Fifteen ml of Dulbecco's phosphate buffered saline (PBS) was added slowly.

Cells were centrifuged at 300xg for 10 minutes. The PBS was discarded, and the cell pellet was gently resuspended in 15 ml of medium, pre-equilibrated to 37 °C and 5% CO_2. Plating density was $1x10^6/cm^2$; standard cell culture plasticware was used. Half of the medium was replaced on days 3, 5 and 7. Colonies of CFU-F were observed on day 10. Full medium changes were performed from that point onwards, three times a week. Cells were passaged at 75-80% confluency, after washing twice with calcium- and magnesium-free PBS followed by a minimal volume of Triple E Select (Thermo Fisher Scientific).

Flow cytometry was performed using a BD Accuri™ C6 flow cytometer. Fluorescent-conjugated antibodies to human CD73, CD90, CD105, HLA-DR, CD45, CD34, CD14 and CD19 were purchased from Becton Dickinson (BD). For each antibody, an isotype control was used. For each flow analysis, 50,000 events were collected and analyzed.

Differentiation into adipocytes, chondrocytes and osteoblasts was achieved using differentiation medias and Matris F plate coating, from Irvine Scientific. Cells were plated at a high density to cause grow arrest and initiate differentiation. For osteogenic and adipogenic differentiation, cells were plated at 30,000 to 40,000 cells/cm^2. For chondrogenic differentiation, cells were plated at 100,000 cells/cm^2. Media was changed three times a week, for four to five weeks. For histological evaluation, adipocytes and chondrocytes were fixed with 4% paraformaldehyde, then washed with water. Adipocytes were stained with Oil Red O; chondrocytes were stained with 0.5% Alcian blue, adjusted to pH 1.0. Osteoblasts were treated with citrate:acetone:formaldehyde fixative, alkaline phosphatase reagents, and then counterstained with neutral red (Sigma AP staining kit).

Results

Over the course of ten to fifteen days, there was a notable change in the appearance of the **PURE PRO®** cells cultured in MSC medium. Initially, the cells were nonadherent and lymphoblastoidic. Over time, cells adhered to the surface of the plastic cell culture flask, then flattened and elongated to form colony-forming units (CFUs). After the first passage, cultures had a uniform fibroblastoid appearance, characteristic of MSCs (Figure 1). The MSCs grew rapidly, with a doubling time of about 24 hours. (The round, bright white circles in this figure are cells undergoing DNA replication and division.) The viability of these MSC cultures was typically between 90-100%, as determined by propidium iodide exclusion.

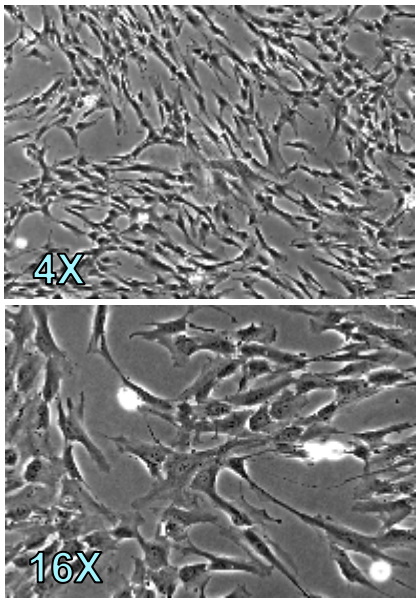

*Fig. 1. Morphology of MSCs derived from **PURE PRO®**. Representative images at 4X and 16X magnification.*

MSCs are characterized by a panel of cell surface markers, as defined by the ISCT[9]. It is well established that MSCs should express CD90, CD73, and CD105, and lack endothelial and hematopoietic markers such as CD14, CD19, CD34 and CD45. Low expression (<1%) of HLA-DR is also characteristic. MSCs derived from **PURE PRO®** showed this classic MSC phenotype.

Flow cytometry analysis is shown in Figure 2. Less than 1% of the cells were positive for CD14, CD19, CD45, CD34, and MHC class I (HLA-DR). The low level of HLA-DR suggests the cells would not be recognized as foreign when transplanted (i.e. would not activate an inflammatory immune response). Cells were uniformly positive (100%) for CD73, and had high levels of CD90 and CD105 (>65%). Interestingly, $CD73^{high}$ MSCs are reported to have better reparative and anti-inflammatory activity than $CD73^{low}$ MSCs.[10]

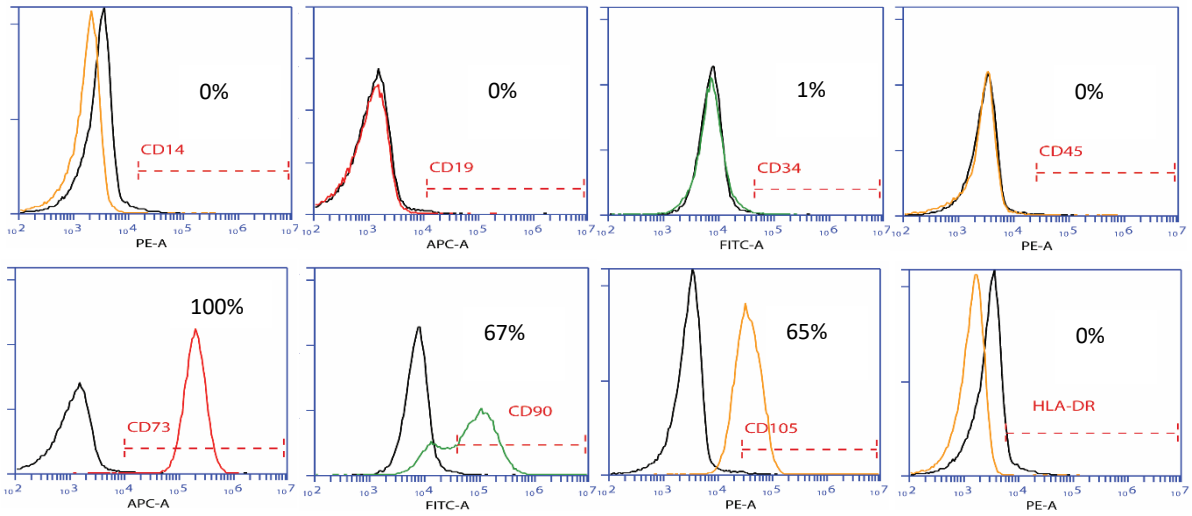

Figure 2. Histograms of flow cytometry results. Cells were cultured for 4 passages, harvested, and labeled with antibodies. Results for the specific antibodies are shown in color: yellow phycoerythrin (PE) for CD14, CD45, CD105 and HLA-DR, green FITC for CD34 and CD90, and red allophycocyanin (APC) for CD19 and CD73. The respective isotype controls are shown in black.

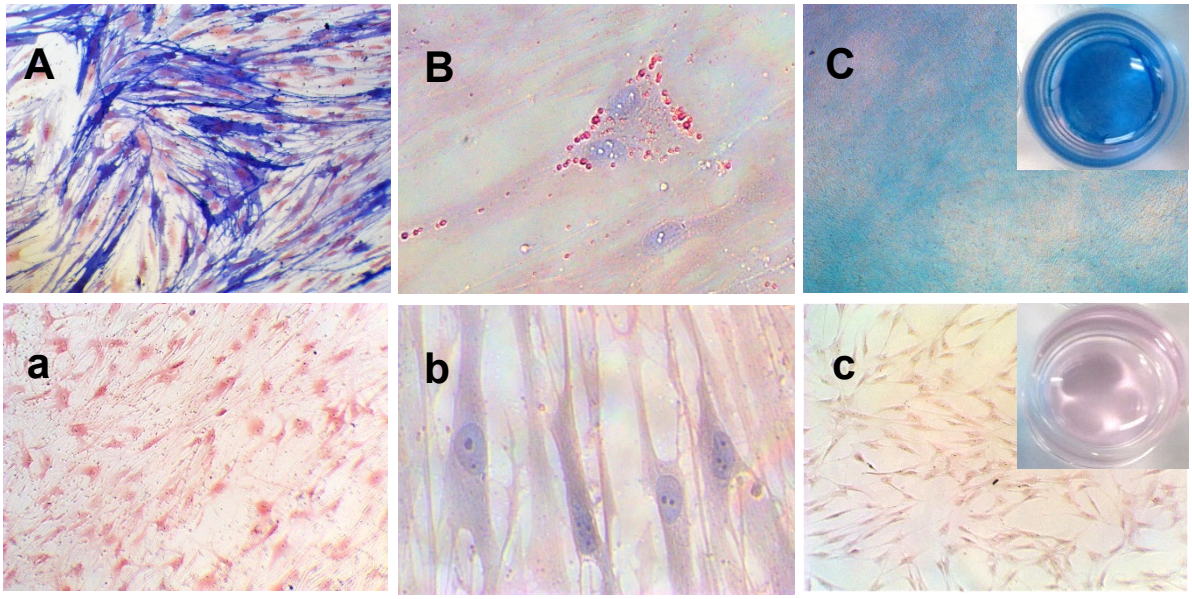

Figure 3. Tri-lineage differentiation of MSCs from PURE PRO®. Osteogenic differentiation, A; adipogenic differentiation, B; chondrogenic differentiation, C. Control cultures (a, b, and c) were maintained in growth medium. Differentiated and control cultures underwent identical staining procedures. Osteogenic differentiation was demonstrated by blue alkaline phosphatase staining (10X magnification). Adipogenic differentiation was demonstrated by accumulation of lipid vacuoles stained by Oil Red O (80X magnification). Chondrogenic differentiation was demonstrated by Alcian blue staining for sulfated mucopolyglycans (10X magnification). Inserts (C and c) are a macroscopic view of the chondrogenic and control cultures.

The ability of **PURE®** product-derived cells to differentiate into adipogenic, osteogenic, and chondrogenic lineages was investigated (Figure 3). Cells were induced to differentiate, while control cultures were maintained in growth medium. Tri-lineage potential was tested by staining for typical lineage markers. Osteogenesis was defined by alkaline phosphatase staining. The diffuse, blue dye indicated alkaline phosphatase enzyme activity throughout the culture. Adipogenesis was observed by staining cytoplasmic lipid droplets with Oil Red O. The fat-soluble red dye revealed many small lipid inclusions throughout the culture; these were obvious at higher magnification. Chondrogenesis was characterized by Alcian blue staining of sulfated mucopolyglycans. Cells maintained in growth medium did not bind the dye, while chondrogenic cultures did. These results demonstrate that MSCs from **PURE®** product can successfully differentiate into multiple cell types: osteoblasts, adipocytes and chondrocytes.

Conclusions

Our investigations put to rest any doubt that MSCs are present in umbilical cord blood, and are present in the **PURE®** product. We show that: 1) MSCs can be isolated from the product, 2) the MSCs have the appropriate cell surface markers as defined by the ISCT, and 3) the cells can self-renew as MSCs, or differentiation into other cell types. Further, the MSCs were isolated after thawing the frozen product; isolation of MSCs was achieved without additional manipulations such as lymphocyte depletion or antibody selection.

Hundreds of publications demonstrate the feasibility of isolating MSCs from cord blood. Still, the abundance of CB-MSC progenitors is contested. Some authors have little success isolating CB-MSCs[11], while others have a success rate of 90% or more[12]. Successful isolation of MSCs depends on many factors: cell number, length of time between cord blood collection and cell culture, medium composition, specific growth factors, etc. Some workers fail to isolate CB-MSCs simply because they do not use optimized culture conditions, or use low-quality starting materials.

MSCs from different tissues share many similarities, but the differences may be important. Some of the CD markers are differentially expressed[13]. There are functional differences as well. CB-MSCs have a greater replicative capacity and can produce a greater number of daughter cells and differentiated progeny. MSCs can be prepared from a patient's bone marrow or fat, but the invasive isolation can be painful and harbors a risk of adverse events. In addition, MSCs from adult sources are aged – having undergone a lifetime of DNA-damage and telomere shortening that neonatal stem cells have not.

Cord blood and products such as **PURE®** may be a preferred source for MSCs, given the availability and convenience of the starting materials. Furthermore, multiple reports show that CB-MSCs have higher proliferative capacity, better anti-inflammatory activity and karyotype stability. Hence, CB-MSCs may have greater suitability for regenerative medicine.

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