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Effect of serum choice on replicative senescence in mesenchymal stromal cells

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Abstract

Background aims. Multipotent mesenchymal stromal cells (MSCs) are promising candidates for innovative cell therapeutic applications. Before their use, however, they usually need to be expanded *in vitro* with serum-supplemented media. MSCs can undergo replicative senescence during *in vitro* expansion, but it is not yet clear how serum supplements influence this process. *Methods.* In the present study, we compared how media supplemented with fetal bovine serum (FBS) or calf serum (CS) affected morphology, proliferation, differentiation, senescence and other functional characteristics of human umbilical cord–derived MSCs (UC-MSCs). *Results.* UC-MSCs cultured in both FBS- and CS-containing media were able to differentiate along osteogenic and adipogenic lineages but ultimately reached proliferation arrest. However, senescence-associated characteristics, such as β-galactosidase activity, reactive oxygen species levels, proliferation rate and gene expression, demonstrate that UC-MSCs grown with FBS have better proliferation potential and differentiation capacity. In contrast, UC-MSCs grown with CS have a higher proportion of apoptotic cells and senescent characteristics. Possible mechanisms for the observed phenotypes include changes in gene expression (Bax, p16, p21 and p53) and cytokine production (interleukin-6 and interleukin-8). *Conclusions.* This study demonstrates that FBS-supplemented media provides a better microenvironment for the expansion of UC-MSCs *in vitro* than CS-supplemented media. This work provides insight into MSCs generation practices for use in basic research and clinical therapies.

Key Words: *calf serum, fetal bovine serum, human umbilical cord–derived mesenchymal stromal cells, replicative senescence*

Introduction

Mesenchymal stromal cells (MSCs) are of tremendous interest for cell-based therapies and transplantation medicine because of their ability to self-renew, differentiate, modulate the immune system and secrete bioactive factors [1–3]. Virtually all post-natal organs and tissues contain MSCs, but the fetal adnexa, which include the umbilical cord (UC) and placenta, is considered an ideal source [4–6]. Despite significant declines in cell proliferation and differentiation capacity over time, UCs remain the principal source of MSCs in studies investigating their use for cell therapy. However, similar to most somatic cells, MSCs have a limited life span *in vitro* [7–10]; after a certain number of cell divisions, MSCs enter replicative senescence and stop proliferating.

In general, MSCs are expanded *in vitro* before therapeutic use. During expansion, serum is frequently added to the defined basal medium to provide a nutritional source and growth factors essential for cell proliferation [11]. This conventional method to isolate and expand MSCs uses basal media supplemented with fetal bovine serum (FBS) or calf serum (CS). FBS and CS are sourced from postnatal bovine. FBS and CS have different physiological status, leading to different substance concentration of FBS and CS, such as energy substrates, cortisol, cholesterol, thyroid hormone analogues, vitamins, amino acids and growth factors. Such differences between the FBS and CS reflect the different physiological developmental stages of bovine fetus and the calf. However, the serum source can affect some MSC characteristics [12–14].

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Therefore, some trials have used alternative serum sources that more closely mimic a physiological environment to delay replicative senescence [15]. However, it is not yet clear whether FBS and CS differentially influence the morphology, proliferative capacity, differentiation potential, function and replicative senescence of expanded MSC populations.

In this study, we compare the effect of culturing human UC-derived MSCs (UC-MSCs) and bone marrow–derived MSCs (BM-MSCs) in FBS or CS. For example, we determined the expression of genes associated with cellular replication and senescence as well as the profile of secreted cytokines from cultured MSCs. We also focused on differentiation, apoptosis and the aging-related index changes of MSCs expanded in these different conditions. This study provides insight into the effects of serum choice on replicative senescence and may help to guide future studies on stem cell and transplant therapies.

Methods

Isolation and culture of MSCs

We obtained human UC samples, with informed consent from donors, from full-term pregnancies at the Chinese People's Liberation Army Hospital [16]. Under sterile conditions, UCs were rinsed three times with phosphate-buffered saline (PBS) to remove contaminating blood. Samples were cut into 2-cm segments, and UC arteries, veins and amnion were removed. The gelatinous tissue was then excised and minced into 0.5- to 1-mm^3 pieces. The minced pieces were placed in a plastic flask, and, after 3 h, Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% bovine serum and 100 U/mL of Pen/Strep was added. Bone marrow samples were diluted and layered on Ficoll-Paque Premium (1.077 g/mL, GE). Samples were then centrifuged at 400*g* for 20 min at 22°C. Mononuclear cells were washed with PBS and plated in DMEM supplemented with 10% bovine serum.

The MSCs were cultured at 37°C in a humidified atmosphere containing 5% CO_2 with medium changes every 3 days. At approximately 80% confluency, the cells were passaged with the use of 0.05% trypsin and seeded in a new culture flack. Subsequently, the cells were cultured in different batches of FBS or CS, respectively (Hangzhou Sijiqing Biological Materials Co, Ltd). The cell morphology of MSCs was observed with the use of a Giemsa stain and photo-documented.

Immunophenotyping

Adherent cells were lifted with the use of trypsin, and aliquots of 1×10^6 MSCs were rinsed twice with cold PBS and suspended in 0.5 mL of PBS. Cells were incubated at 4°C for 30 min with the following fluorescein isothiocyanate (FITC)-conjugated or phycoerythrin (PE)-conjugated antibodies (eBioscience): cluster of differentiation (CD)34, CD45, CD73, CD90, CD105 and human leukocyte antigen (HLA)-DR. Human FITC–immunoglobulin (Ig)G1 and PE-IgG1 were used as isotype controls. After incubation, cells were washed twice with PBS and analyzed by means of fluorescence-activated cell sorting (FACScan, BD Bioscience), and the data were analyzed with the use of FlowJo 7.6 (Tree Star Inc) software.

Proliferation assays

For the evaluation of cumulative population doubling (CPD), cells were seeded, in triplicate, into T75 culture flasks at a density of 3000 cells/cm^2. When the flasks reached 80% confluency, the cells were counted and re-plated at the original density. The population doubling (PD) was calculated by use of the formula $PD = lg2/(lgN_t - lgN_0)$, where N_0 = number of cells seeded and N_t = number of cells counted at confluence. The CPD was calculated by adding the PD obtained at each successive passage. Meanwhile, the growth curve of MSCs was determined during the same culturing process. For the growth curves, cells were seeded, in triplicate, into 24-well plates at a density of 1×10^4 cells per well. At specified times, 1, 2, 3, 4, 5 and 6 days, the cells were lifted with trypsin and counted.

Cell proliferation was also determined with the bromodeoxyuridine (BrdU) Cell Proliferation Detection Kit (Keygentec), following the manufacturer's instruction. In the logarithmic growth phase, BrdU was added at a final concentration of 30 μmol/L. After 7 to 8 h, cells were rinsed twice and incubated on ice for 2 min. Cells were washed and then were incubated in working liquid at 37°C for 30 min before a final wash. Cells were then suspended in 200 μL of staining buffer with 5 μL of a PE-BrdU antibody and incubated for 30 min at 4°C without light. Samples were analyzed with the use of a FACScan flow cytometer (Becton-Dickinson).

For cell cycle analysis, MSCs were harvested with trypsin, washed twice with cold PBS and fixed with 70% ethanol at 4°C for 12 h. Cells were then washed twice with PBS followed by incubation at 37°C for 30 min with propidium iodide (PI, Sers) staining solution (50 μg/mL) and RNase A (0.1 mg/mL; Sigma) and analyzed by means of FACScan. The data were analyzed with the use of Modfit LT 4.0 software.