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MESENCHYMAL STROMAL CELLS

Aging of bone marrow– and umbilical cord–derived mesenchymal stromal cells during expansion

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Abstract

Background aims. Mesenchymal stromal cells (MSCs) are used as experimental immunotherapy. Extensive culture expansion is necessary to obtain clinically relevant cell numbers, although the impact on MSCs stability and function is unclear. This study investigated the effects of long-term *in vitro* expansion on the stability and function of MSCs. *Methods.* Human bone marrow–derived (bmMSCs) and umbilical cord–derived (ucMSCs) MSCs were *in vitro* expanded. During expansion, their proliferative capacity was examined. At passages 4, 8 and 12, analyses were performed to investigate the ploidy, metabolic stability, telomere length and immunophenotype. In addition, their potential to suppress lymphocyte proliferation and susceptibility to natural killer cell lysis was examined. *Results.* BmMSCs and ucMSCs showed decreasing proliferative capacity over time, while their telomere lengths and mitochondrial activity remained stable. Percentage of aneuploidy in cultures was unchanged after expansion. Furthermore, expression of MSC markers and markers associated with stress or aging remained unchanged. Reduced capacity to suppress CD4 and CD8 T-cell proliferation was observed for passage 8 and 12 bmMSCs and ucMSCs. Finally, susceptibility of bmMSCs and ucMSCs to NK-cell lysis remained stable. *Conclusions.* We showed that after long-term expansion, phenotype of bmMSCs and ucMSCs remains stable and cells exhibit similar immunogenic properties compared with lower passage cells. However, immunosuppressive properties of MSCs are reduced. These findings reveal the consequences of application of higher passage MSCs in the clinic, which will help increase the yield of therapeutic MSCs but may interfere with their efficacy.

Key Words: *culture expansion, immunogenicity, immunomodulation, mesenchymal stromal cell*

Introduction

Mesenchymal stromal cells (MSCs) are increasingly used as an experimental therapy for a range of immunological and degenerative diseases [1–5] because of their immunomodulatory properties [6–10]. Clinical trials use MSCs isolated from different tissues [11], most frequently bone marrow–derived (bmMSCs) and umbilical cord–derived (ucMSCs) MSCs [12,13]. They are used in doses ranging from $1–2 \times 10^6$ MSCs/kg body weight with a frequency of 1 to more than 10 infusions per clinical trial. Because the frequency of MSCs in tissues is very low and the amount of tissue available is limited, MSCs need to be expanded *ex vivo* before application to obtain sufficient numbers. Depending on the origin of the MSCs, the age of the donors may vary: ucMSCs originate from newborns, whereas the age of bmMSC donors can vary widely. Notably, there is a positive correlation between donor age and the accumulation of mutations in human adult stem cells [14], although it is unclear whether bmMSCs and ucMSCs age during culture expansion and whether this affects their immunomodulatory therapeutic potential.

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The use of low-passage MSCs for therapy is currently preferred to a higher passage MSCs [15,16] because of the impact of extended passage on immunomodulatory function. Moreover, studies that focused on the therapeutic potential of MSCs for regeneration have shown that the differentiation potential of MSCs is impaired after long-term *in vitro* culturing [17–20]. Notably, under conditions of stress, such as inflammation, MSCs up-regulate their human leukocyte antigen (HLA) type I and II expression, rendering them more immunogenic [21], and they also up-regulate their programmed death-ligand 1 (PD-L1) expression [22], which is also up-regulated by aging T cells [23].

Systematic evaluation of immunomodulatory and genetic stability over passage of MSCs will definitively determine the magnitude of this effect and also define whether there is an optimal passage for immunomodulation. This will standardize MSC therapy and balance the benefits of expansion, giving more doses per MSC donor, with immunomodulatory exhaustion.

Therefore, in this article, we report the effects of prolonged expansion on the stability and function of bmMSCs and ucMSCs. Through analysis of the surface marker expression on bmMSCs and ucMSCs at passage 4, 8 and 12, we examined the stability of the MSC immunophenotype during prolonged culture. In addition, we evaluated up to which culture stage their proliferative capacity was maintained, which is of great importance for determining the number of potential therapeutic doses of MSCs that can be obtained from a particular cell donor. To investigate whether prolonged culture would have an effect on aging parameters, telomere length, mitochondrial activity or the number of aneuploid cells in the cultures were determined. Because aging may have effects on the functionality of MSCs, the capacity of passage 4, 8 and 12 bmMSCs and ucMSCs to inhibit lymphocyte proliferation was tested. Finally, to examine whether prolonged culture expansion would affect the immunogenicity of MSCs, the susceptibility of bmMSCs and ucMSCs to trigger natural killer (NK) cytotoxic activity was evaluated.

Methods

Culture expansion of bmMSCs and ucMSCs

Human bone marrow samples were collected from healthy adult donors (n = 3) with written informed consent according to the Ethics Ref. C.A.02/08. Isolation of $CD362^+$ bmMSCs was according to methods described in Elliman *et al.* (manuscript in progress). Briefly, mononuclear cells (MNCs) were isolated by Ficoll density gradient centrifugation (GE Health Care Bio-Sciences) and ACK Lysis Buffer (Life Technologies) used for erythrocyte lysis (n = 3). MNCs were then analyzed for expression of CD235-eFluor 450 (clone 6A7M, dilution 1:1000, eBioscience), CD45-FITC (fluorescein isothiocyanate; clone H130, dilution 1:25), CD271-PE (clone ME20.4-1.H4, dilution 1:50, Miltenyi Biotec) and CD362-APC (clone 305515, dilution 1:50, R&D Systems), and the viability dye Sytox Blue was used to exclude dead cells (as per manufactures instructions, Life Technologies). Using appropriate controls including fluorescence minus one (FMO) sort gates were assigned and the $CD362^+$ $CD271^+$ population was sorted using a BD FACS Aria (BD Biosciences). The mean number of $CD362^+$ $CD271^+$ cells isolated from the donors used in this study was 5419 ± 2359 (mean ± SEM, n = 3). Cells were plated, expanded in culture and cryopreserved at passage 2 for shipment to Erasmus Medical Center.

Human umbilical cord tissue was collected from virally screened healthy donors by Tissue Solutions Ltd. (all cord tissues provided by Tissue Solutions are obtained according to the legal and ethical requirements of the country of collection, with the approval of an ethics committee (or similar) and with anonymous consent from the donor). In the work reported here, umbilical cord tissue (n = 3) was collected from Cesarean deliveries, and all tissues were transported for processing to Orbsen Therapeutics Ltd. in AQIX solution at 4°C. Isolations were performed within 48 h post-birth. Briefly, umbilical cord tissue was washed, and the whole tissue was manually dissociated before additional enzymatic digestion in a MEM Alpha (Gibco, ThermoFisher) enzyme cocktail containing Collagenase 1 (2 mg/mL), Hyaluronidase 1 (1 mg/mL) and DNase (0.1 mg/mL) (Sigma Aldrich) for a maximum of 2 h at 37°C. Once a single cell suspension was obtained by filtration (100 μm), cells were stained with CD362-APC (clone 305515, dilution 1:50, R&D Systems). After 30 min at 4°C, the cells were washed and resuspended in MACs buffer 80 μL/10^7 cells with the addition of anti-APC beads (Miltenyi Biotec) at a concentration of 20 μL/10^7cells, the cells were incubated with beads for 15 min at 4°C. The $CD362^+$ cells were then isolated using MS MACs column as per manufactures instructions (Miltenyi Biotec). Each cell fraction was counted, seeded for expansion and cryopreserved at passage 2 for shipment to Erasmus Medical Center.

BmMSCs and ucMSCs were thawed and cultured in minimum essential medium Eagle alpha modification (MEM-α; Sigma-Aldrich) containing 2 mmol/L L-glutamine (Lonza), penicillin/streptomycin solution (100 IU/mL penicillin, 100 IU/mL streptomycin; Lonza) and supplemented with 15% fetal bovine serum (Lonza) and 1 ng/mL basic fibroblast growth factor (bFGF, Sigma-Aldrich) and kept at 37°C, 5% CO_2 and 20% O_2.

Every 7 days, bmMSCs and ucMSCs were passaged using 0.05% trypsin-ethylenediaminetetraacetic acid (Life Technologies). The cells were seeded at in density of 1×10^5 cells/175 cm^2, and the amount of