Enhancing *ex vivo* expansion of cord blood-derived unrestricted somatic stem cells for clinical applications

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Abstract

Objectives: To study effects of serum-containing medium (SCM) versus serum-free medium (SFM) and influence of seeding density, on rate of expansion of cord blood (CB) unrestricted somatic stem cells (USSCs), as a prerequisite for evaluating their therapeutic potential in ongoing clinical trials.

Material and methods: Isolation, propagation and characterization of USSCs from CB samples were performed and followed by their passage 3 culture in SCM and SFM, at cell densities of 5, 50, 500 and 5000 cells/cm².

Results: The cells were CD44⁺, CD90⁺, CD73⁺, CD105⁺, CD34[−], CD45[−], and HLA-DR, with *Oct4* & *Sox2* gene expression; they were differentiated into osteoblasts and adipocytes. USSCs cultured in SCM had significantly higher population doubling levels ($P < 0.01$) than those cultured in SFM. Those cultured in SCM at 5 cells/cm² and those cultured in SFM at 50 cells/cm² had significantly higher population doubling ($P < 0.01$) levels than those cultured at higher cell densities.

Conclusions: For scaling up of USSCs from 106 (?) to 1012 (?) in 6 weeks, culturing of CB-derived cells of early passage ($\leq P3$) in SCM at low cell seeding density (5 cells/cm²) is suggested for increasing cell count with lower passaging frequency, followed by culture of expanded USSCs at 50 cells/cm² in SFM, to avoid undesirable effects of bovine serum in clinical applications.

Introduction

Cord blood (CB)-derived unrestricted somatic stem cells (USSCs), adherently growing fibroblast-like cells, were identified in CB, similar to bone marrow-derived mesenchymal stem cells (MSCs). However, they differ in their ability to expand extensively (up to 10¹⁵ cells) without losing multipotency in culture (1).

Unrestricted somatic stem cells are a somewhat underexplored population of adult stem cells, which may provide advantages over the well-characterized haematopoietic stem-cell population. Ready availability from CB, ease of expansion *in vitro*, simple isolation due to their adherence to plastic, ability to evade rejection and their multipotentiality in differentiation, make them ideal for clinical applications (2,3).

For most of clinical work envisioned, a very large number of USSCs/MSCs is required (4,5). Furthermore, large-scale commercialization of MSCs, where cells from single donors are expanded into thousands of individual doses for use in the clinic, is emerging (6). A wide gap exists, however, between numbers of USSCs that can be obtained from CB and numbers of USSCs needed for implantation for tissue regeneration. Thus, it is essential to culture and expand their numbers *in vitro*, before being able to put them to therapeutic use.

Unrestricted somatic stem cells have been found to reach high numbers after long culture periods and sequential passaging, but adverse effects of repeated passaging and long culture times highlight challenges in maintaining them in the undifferentiated state (7). It is desirable to know an ideal number of passages which maximizes stem-cell purity, potential, and protection conferred, but this might possibly limit their *ex vivo* expansion before therapeutic use (7). To reach a compromise between USSC number, repeated passaging and culture duration, we have conducted the investigation described here.

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The aim of the study was determination of the most favourable culture media and seeding density, to drive USSC population to expand extensively in one passage, preferably an early one (P3 or less), to generate cell numbers suitable for clinical application without losing the cells' differentiation potential.

Materials and methods

Collection of cord blood

Human CB was obtained at full-term delivery (38–40 weeks) caesarean-sectioned patients after informed written consent, as approved by the institutional review board at Theodor Bilharz Research Institute (TBRI), Cairo, Egypt. Under complete aseptic conditions, the umbilical cord was clamped from the baby's side, then washed in 70% ethanol. The umbilical vein was punctured using a 50 ml heparinized (5000 IU/ml heparin) syringe with a wide bore needle, to avoid blood haemolysis and to provide good negative pressure (8). After collection, blood was transferred to the laboratory at -22°C and processed immediately in the tissue culture lab. Collected CB was tested for hepatitis C virus antibodies, hepatitis B surface antigen and human immunodeficiency virus antibodies before manipulation, using rapid tests for HBV, HCV and HIV (Acon Laboratories, San Diego, CA, USA). Also, complete blood count was performed using Beckman Coulter (Nyon, Switzerland) apparatus.

Generation and expansion of USSCs from CB

Isolation and expansion of USSCs from CB were performed according to Kögler *et al.* (1). Briefly, the mononuclear cell fraction was obtained by Ficoll (Biochrom, Berlin, Germany) gradient separation at 850 g for 20 min at 20°C after being added at 1:1.5 (1 ml Ficoll:1.5 ml blood), followed by red blood cell lysis using ACK (ammonium-chloride-potassium) lysing buffer. Separated mononuclear cells were cultured at 1×10^6 cells per cm^2 into tissue culture flasks in serum-containing medium (SCM), consisting of complete Dulbecco's modified Eagle's medium (DMEM)/low glucose (Lonza, Visp, Switzerland) supplemented with 30% foetal bovine serum (FBS), (Invitrogen Gibco, Carlsbad, CA, USA), 1% penicillin/streptomycin (Biochrom), 1% L-glutamine (Lonza) and dexamethasone 10^{-7} M (Sigma-Aldrich, Steinheim, Germany), and incubated at 37°C in 5% CO_2 and 90% humidified atmosphere. Ten days later, flasks were inspected for colony formation of adherent cells with MSC-like morphology. When colonies were detected, SCM medium was changed twice weekly, without dexamethasone. After a number of days, monolayers of adher-

ent fibroblast-like USSCs were formed. This was defined as the induction phase and was called passage 0. On reaching around 90% confluence, adherent USSCs were detached using 2.5% trypsin (Euro-lone, Milan, Italy). After trypsinization, they were then cultured at 5000 cells/ cm^2 to provide the P1. Cryopreservation of portions of total numbers of cells, in liquid nitrogen, was performed starting from P2. Trypsinization and passaging were repeated until the cells ceased further proliferation.

Immunophenotypic analysis of USSCs

This step was performed according to Hartmann *et al.* (9). Cells were stained with fluorescein isothiocyanate (FITC) mouse anti-human CD44, CD73, CD 90, HLA-DR (BD Biosciences, San Jose, CA, USA), FITC mouse anti-human CD105 (R&D Systems, Minneapolis, MN, USA), FITC mouse anti-human CD34, CD45 coupled with phycoerythrin (BD Biosciences) for 30 min (concentrations of monoclonal antibodies added were according to the manufacturers' instructions). Cells were analysed using flow cytometry (Beckman Coulter Epics XL-MCL). As control, unstained cells were applied first, to exclude effects of autofluorescence of cultured cells.

Gene expression analysis of USSCs

Molecular analysis of USSCs was performed according to Zaibak *et al.* (10). Total RNA was extracted from 5 to 8×10^6 trypsinized USSCs using RNeasy Mini Kit (Qiagen, Hilden, Germany). Concentration and purity of extracted RNA were assessed using NanoDrop 2000 spectrophotometry (Thermo Scientific, Wilmington, DE, USA). Reverse transcripts were prepared from extracted RNA using a high-capacity cDNA kit (Applied Biosystems, Foster City, CA, USA). Expression of the following genes was detected by real-time PCR (Step one; Applied Biosystems): *Oct4* (5' TCTCGCCCCCTCCA GGT; 3' GCCCACTCCAACCTGG), *Sox2* (5' AGCTA CAGCATGATGCAGGACC; 3' CTGGTCATGGAGTT GTACTGCAGG), normalized to *GAPDH* (5' ATGGAG AAGGCTGGGGCTC, 3' AAGTTGTCATGGATGACC TTG) as reference gene, using QuantiTect SYBR Green PCR kit (Qiagen).

Osteogenic differentiation of USSCs

Osteogenic differentiation was performed according to Kögler *et al.* (11). P3 USSCs were seeded at 5×10^3 cells/ cm^2 in 6-well plates, and were cultured in SCM. When they had reached approximately 80% confluence, medium was replaced with osteogenic differentiation medium (85% differentiation basal medium, 10% mes-

enchymal cell growth supplement, 2% L-glutamine, 1% penicillin/streptomycin, 0.5% dexamethasone (10^{-4} M), 0.5% ascorbate and 1% B-glycerolphosphate) (Lonza). Medium was changed twice weekly for 2–3 weeks. After 21 days, differentiated cells were stained with alizarin red.

Adipogenic differentiation of USSCs

To assess adipogenic differentiation, P3 USSCs were seeded at 5×10^3 cells/cm² in 6-well plates, and cultured in SCM. When they reached approximately 80% confluence, adipogenic differentiation was induced by alternately changing adipogenic induction medium (supplemented with 10 mg/ml insulin, 0.5% dexamethasone (10^{-4} M), 50 mM indomethacine and 500 mM 3-isobutyl-1-methyl-xanthine, 10% FBS and 1% penicillin/streptomycin) and adipogenic maintenance medium (supplemented with 10 mg/ml insulin, 10% FBS and 1000 IU/ml penicillin/streptomycin) (Lonza) every 3–4 days (12). Negative controls (undifferentiated USSCs) were kept in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine. On the 21st day, adipogenic differentiation was assessed by oil red-O (Gibco) staining.

Variable factors affecting USSC proliferation

P2 USSCs were retrieved from liquid nitrogen and cultured at 5000 cells/cm² in SCM for 3 days until reaching 90% confluence. They were then harvested and a portion of them were cultured as P3, either in SCM or in serum-free medium (SFM; Mesencult) (Stemcell Technologies, Vancouver, BC, Canada), at 4 different seeding densities (5, 50, 500 and 5000 cells/cm²). Population doubling (PD) and culture days were compared between the different cultures. When cells reached 90–100% confluence, they were trypsinized and counted. PD was calculated according to Cristofalo *et al.* (13), using the following formula:

$$PD = \frac{\log(NH) - \log(N1)}{\log(2)}$$

where N1 is the first cell count and NH is the cell count after reaching 90–100% confluence.

Statistical analysis

Data were analysed using a statistical package (SPSS for Windows 18.0, SPSS, Chicago, IL, USA) and expressed as mean $\pm$ SEM; *P* less than 0.05 was considered to be statistically significant. Mean values of USSC PDs and culture days between different variables were compared using the analysis of variance (ANOVA) method.

Results

Isolation and expansion of USSCs

Cells were isolated from 10 of 22 CB samples (45%); cultures varied from 70 to 90 days (mean = 77 days) and passage number ranged between 8 and 12 (mean = 10 passages).

Characterization of the USSCs

Morphology. The cells were of typical MSC morphology – adherent and fibroblastoid, spindle-shaped and growing in a monolayer (Fig. 1).

Immunophenotyping. Cells expressed high levels of adhesion marker CD44, typical mesenchymal markers CD90 and CD73, and endolgin receptor CD105, whereas they did not express haematopoietic lineage marker CD34, leucocyte common antigen CD45, nor human leucocyte antigen class II, HLA-DR, as shown in Table 1 and Fig. 2.

Gene expression analysis. Cells expressed of transcripts of *Oct4*, and *Sox2*, considered to be core transcription factors in early embryo development and pluripotency maintenance of embryonic stem cells. Relative quantification values of expressed genes are shown in Fig. 3.

Differentiation potentials of the USSCs

Cells were induced to osteogenic and adipogenic differentiation. Osteogenic differentiation potential was confirmed by formation of mineralized matrix as revealed

Figure 1. Unrestricted somatic stem cells showing classical mesenchymal stem cells morphology of adherent fibroblastoid spindle-shaped cells growing in a monolayer.

Table 1. Percentage of immunophenotype markers expression of USSCs.

Immunophenotypes	USSCs of P3 (%)
CD73	99.90
CD44	91.50
CD105	91.30
CD90	94.60
CD34	00.34
CD45	02.39
HLA-DR	02.76

USSCs, unrestricted somatic stem cells.

by alizarin red staining (Fig. 4a), while adipogenic differentiation was confirmed by accumulation of neutral lipid vacuoles revealed by oil red-O stain (Fig. 4b).

Effect of seeding density and culture media on USSC proliferation

Effects of seeding density on proliferation rate of the USSCs revealed that their PD when cultured at 5 cells/cm² in SCM was significantly higher ($P < 0.01$) than those cultured at higher densities, while, PD of USSCs cultured at 50 cells/cm² in SFM was significantly higher ($P < 0.01$) than that of those cultured at other cell densities (Fig. 5).

Culture days of USSCs grown in SCM at 5 cells/cm² were comparable to those cultured at higher densities. However, cells cultured in SFM at 5 and 50 cells/cm² needed significantly longer ($P < 0.001$) than those cultured at higher cell densities (500, 5000 cells/cm²).

Regarding effects of culture media on USSC expansion, we found that cells cultured in SCM had significantly higher PD ($P < 0.001$) and required fewer culture days ($P < 0.01$) than those cultured in SFM at 5 and 50 cells/cm² (Figs 6,7).

Counts of harvested cells from culture of 10⁶ USSCs in either SCM or SFM at different cell densities

Expansion potential of 10⁶ USSCs cultured in SCM at 5, 50, 500 and 5000 cells/cm² was 10¹⁰, 1.2 × 10⁹, 10⁸ and 0.5 × 10⁷, respectively, while expansion potential of USSCs cultured in SFM was 2 × 10⁸, 5 × 10⁸, 7 × 10⁷ and 1.2 × 10⁷ at 5, 50, 500 and 5000 cells/cm², respectively, as shown in Table 2.

Discussion

Cord blood-derived USSCs represent early mesodermal precursors, and to become MSCs is one of their various

differentiation fates (14). Being early progenitors of MSCs, USSCs have been made to expand to high levels in long-term culture systems, permitting their large-scale production (1,14).

Stem-cell therapy has emerged as an exciting new area of medicine and surgery. Plasticity of progenitor cells has resulted in positive remodelling and regeneration of viable tissues in liver, brain, heart and other organ systems (15). Among the many sources of adult stem cells, CB-derived USSCs have shown particular promise (3).

In our study, CB-USSCs had morphological characteristics and the immunophenotypic profile of MSCs, being adherent fibroblastoid cells, expressing adhesion marker CD44, typical mesenchymal markers CD90 and CD73 and endoglin receptor CD105, whereas they did not express haematopoietic lineage marker CD34, leucocyte common antigen CD45 and human leucocyte antigen class II (16). The cells highly expressed transcripts of *Oct4* and *Sox2*, these being considered core transcription factors in maintenance of pluripotency in embryonic stem cells (17,18). Moreover, our USSCs were successfully induced to differentiate into both osteogenic and adipogenic lineages.

Unrestricted somatic stem cells/MSCs hold great promise as therapeutic agents in regenerative medicine and for autoimmune diseases (19), and *ex vivo* expansion is a prerequisite for evaluating their therapeutic potential in ongoing clinical trials. For optimal culture conditions, several factors influence USSC expansion while maintaining differentiation potential, including culture media, oxygen tension, serum supplements, growth factors and cell plating density (20). These are expected to act by dramatic reduction in unwanted side-effects caused by enzymatic cell passaging, leading to faster and longer cell number expansion and maintenance of efficient proliferation rates.

Serum has been found to be the most uncertain factor in expansion of clinical grade USSCs, considering batch-to-batch variability, possibility of viral contamination and anticipated immune rejection of transplanted cells conjugated with bovine proteins (21). Serum-free culture medium, as an alternative to SCM, has been implemented for potentially better safety of cell-based therapy. Use of growth factors as culture supplements instead of FBS offers the most promising alternative, as SFM consists of a blend of essential amino acids, inorganic salts and other components, along with an optimized mix of recombinant human growth factors, platelet-derived growth factor, fibroblast growth factor and transforming growth factors (22,23).

Previous studies have shown the ability of USSCs to expand extensively after several passages (1). However,

Figure 2. Immunophenotype analysis of unrestricted somatic stem cells.

these stem cells senesce and lose proliferation and differentiation potential with increasing time in culture and repeated passaging (24–26). The study described here was designed to determine influence of log fold reduced USSC seeding (5000, 500, 50, 5 cells/cm²), as well as effects of SCM versus SFM, on clinical-scale expansion of P3 USSCs, being an early rapidly proliferating passage.

When studying effects of SCM versus SFM on USSC PD and culture duration, we found that cells cultured in SCM had significantly higher PD ($P < 0.01$) and shorter culture periods ($P < 0.001$) at 5 and 50 cells/cm² than those cultured in SFM at the same densities. Several protocols have been proposed for expansion of MSCs/USSCs in medium containing 10–30% FBS (11,27–29). Chase *et al.* (23) reported that DMEM sup-

Figure 3. Relative quantification values (RQ) of *Oct4*, *Sox2* genes of passage 3 unrestricted somatic stem cells. Where $RQ = 2^{-\Delta\Delta C_T}$, $\Delta\Delta C_T = [\Delta C_T (\text{unknown sample}) - \Delta C_T (\text{calibrator sample})]$, $\Delta C_T (\text{unknown sample}) = [C_{T \text{ GI}} (\text{unknown}) - C_{T \text{ GR}} (\text{unknown})]$, $\Delta C_T (\text{calibrator sample}) = [C_{T \text{ GI}} (\text{calibrator}) - C_{T \text{ GR}} (\text{calibrator})]$, where C_T is the number of cycles needed for the fluorescence to reach a specific threshold level of detection, GI is the gene of interest, and GR is the reference gene.

plemented with FBS made up a traditional basal medium providing robust undifferentiated USSC expansion. In addition, Ayatollahi *et al.* (30) compared three concentrations of FBS (5%, 10% and 15%) and found that MSCs cultured in DMEM containing 15% FBS expanded rapidly for up to 10 passages, while those cultured in DMEM containing 5% FBS gradually lost proliferation capacity.

Regarding effects of seeding density on proliferation rate (PD) of USSCs, we found that USSCs cultured at

the lowest seeding density (5 cells/cm²) had the highest PD ($P < 0.01$) when cultured in SCM compared to those cultured at higher seeding densities. Cell seeding at lower density has been said to allow for avoiding frequent enzymatic disruption of cell–cell contacts and to reduce cell loss connected to additional steps of cell harvesting and reseeded (31). As for USSC cultured in SFM, we found that the highest PD ($P < 0.01$) was achieved at 50 cells/cm² when compared to other cell densities. Similar to our results, Sekiya *et al.* (32) and Chase *et al.* (23) suggested that cells grown in SCM can be grown optimally at very low seeding densities, whereas cells in SFM appear to perform better at higher seeding densities.

Low plating density is said to result in higher yields and faster expansion of USSCs/MSCs due to their colonogenic properties at low-density cultures, which provide adequate surfaces for extensive replication (29,32). Higher growth potential at lower seeding densities may also be due to more availability of nutrients per cell, while lower growth rate of cells seeded at higher densities could be due to contact inhibition (33).

Ability to scale up USSC population numbers is critical for any viable cell therapy. Human USSCs must be replated after reaching 70–90% confluence because contact inhibition reduces cell proliferation rate. With repeated passaging, USSCs lose pluripotency and proliferation capacity, partly because of exposure to enzymes such as trypsin, that degrade cell surface proteins. Whereas early USSCs (≤ 5 passages) preserve pluripotency, late passage cells (≥ 15) are able to differentiate only into adipocytes (34).

It has been recommended by the Food and Drug Administration that ‘minimally manipulated’ cells be

Figure 4. (a) Osteogenic differentiation of unrestricted somatic stem cells as shown by alizarin red staining of layered cell clusters, surrounded by matrix-like substance with calcium deposits. (b) Adipogenic differentiation of unrestricted somatic stem cells demonstrated as oil-red staining of lipid vesicles.

Figure 5. Effect of different seeding densities (5, 50, 500, 5000 cells/cm²) on population doubling of unrestricted somatic stem cells cultured in either serum-containing medium (a) or serum-free medium (b). Box plots represent the interquartile range; top and bottom of the box are, respectively, 25th and 75th percentiles. Line across the box is the median. Lower and upper values are indicated by the whiskers. *Out range data: (a) Serum-containing medium population doubling at 5 cells/cm² is significantly higher ($P < 0.01$) than at 50, 500, 5000 cell/cm²; (b) Serum-free medium population doubling at 50 cells/cm² is significantly higher ($P \ll 0.01$) than at 5, 500, 5000 cell/cm².

Figure 6. Effect of culture media, serum-containing medium versus serum-free medium, on population doubling (mean $\pm$ SEM) of unrestricted somatic stem cells cultured at different seeding densities (5, 50, 500, 5000 cells/cm²). * $P < 0.01$.

Figure 7. Effect of culture media, serum-containing medium versus serum-free medium on culture days (mean $\pm$ SEM) of unrestricted somatic stem cells cultured at different seeding densities (5, 50, 500, 5000 cells/cm²). * $P < 0.01$.

used for human clinical trials. In this regard, attempts are being made to develop an efficient production system to produce clinically relevant numbers of human

Table 2. Expected expansion potential of 10^6 USSCs cultured in either SCM or SFM at various cell densities.

Cell density/cm ²	Count of USSCs retrieved after trypsinization	
	SCM	SFM
5	10^{10}	2×10^8
50	1.2×10^9	5×10^8
500	10^8	7×10^7
5000	0.5×10^7	1.2×10^7

USSCs, unrestricted somatic stem cells; SCM, serum-containing medium; SFM, serum-free medium.

Cell count of USSCs retrieved after reaching 90% confluency in cultures is calculated from the following equation: $\log x = (PD \times \log_2) + \log_{10}^6$, where x is the number of USSCs harvested on reaching 90% confluency, and PD is the mean population doubling of cultures at various cell densities.

USSCs/MSCs in relatively shorter culture periods of time with lower passage numbers (35). In this study, when we estimated expected cell count of 10^6 USSCs cultured for one passage only in either SCM or SFM at our culture densities, we found scale-up of USSCs from 10^6 to 10^{10} in 2 weeks when plated at 5 cells/cm² in SCM, with 12-, 100- and 2000-fold increase in counts compared to those cultured at 50, 500 and 5000 cells/cm², respectively. As for culturing USSCs in SFM, maximum number of cells was retrieved when they were cultured at 50 cells/cm² with scaling up of their number from 10^6 to 5×10^8 in 4 weeks.

In conclusion, as progenitor cells senesce and lose differentiation potential over increasing time in culture and passage number (36), and on the basis of optimizing cell culture conditions of USSCs to increase their number to a high level suitable for clinical applications, the

following two-step scenario is recommended for scaling up of the cells from 10^6 to 10^{12} in 6 weeks only. First, is culturing CB-derived USSCs of early passage ($\leq P3$) in SCM (30% FBS/DMEM) at low cell seeding density (5 cells/cm²) to allow for increasing cell count with lower passaging frequency and shorter culture period, to avoid unwanted side-effects of repeated trypsinization, and to maintain pluripotency. The second step is culture of expanded USSCs, on demand, at 50 cells/cm² in SFM (Mesencult), to avoid undesirable effects of FBS in clinical applications. Efficiency of this two-step procedure for clinical scaling up of USSCs could facilitate their implementation in cell-based therapies.

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Disclosure of interest

The authors declare no conflict of interest.

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