Cell Biology International

Cell Biol. Int. (2012) 36, 415–418 (Printed in Great Britain)

Short Communication

Quantity and proliferation rate of mesenchymal stem cells in human cord blood during gestation

Lan-Lan Ma*,†, Fan-Biao Meng‡,§, Ping Shi*, Gang Li‡,§,¶[1] and Xi-Ning Pang[1]*

* Key Laboratory of Cell Biology of China Ministry of Public Health, Department of Stem Cells and Regenerative Medicine, China Medical University, Shen Yang, People's Republic of China

† Physiology Center, China Medical University, Shen Yang, People's Republic of China

‡ Stem Cell and Regeneration Program, Li Ka Shing Institute of Health Sciences, Prince of Wales Hospital, The Chinese University of Hong Kong, Shatin, Hong Kong, SAR, People's Republic of China

§ Department of Orthopaedics and Traumatology, Prince of Wales Hospital, The Chinese University of Hong Kong, Shatin, Hong Kong, SAR, People's Republic of China

¶ MOE Key Laboratory of Regenerative Medicine, School of Biomedical Sciences, Basic Medical Sciences Building, The Chinese University of Hong Kong, Shatin, Hong Kong, SAR, People's Republic of China

Abstract

UCB (umbilical cord blood) as a resource of MSCs (mesenchymal stem cells) is widely accepted, but the quantity and characteristics of UCB-MSCs from different gestational ages have not been well studied. We have quantified the number of MSCs in UCB at different gestational ages using a multi-colour flowcytometer and compared the cell proliferation rates of these UCB-MSCs. Defining MSCs as CD44+/CD105+/CD34−/CD45 population, their numbers declined in the UCB at the gestational age. Proliferation rates were significantly higher in UCB before term than at full term. Non-full term UCB samples may be a better source of MSCs.

Keywords: flow cytometry; human umbilical cord blood; mesenchymal stem cell (MSC)

1. Introduction

UCB (umbilical cord blood) contains abundant stem cells that can be successfully isolated for use in treating haematopoietic disorders. MSCs (mesenchymal stem cells) are also present in UCB and have similar phenotypes as bone marrow-derived MSCs with multi-lineage differentiation potential (Kern et al., 2006; Manca et al., 2008). Compared with bone marrow MSCs, UCB-MSCs are readily available, easy to harvest and evoke little ethic controversy. However, isolation of MSCs from full-term UCB has not always been successful due to the sparse population at full-term UCB. The frequency of MSCs in full-term UCB is only 0.00003% of nucleated cells (Campagnoli et al., 2001). The number of MSCs from bone marrow declines with age (Stenderup et al., 2003), but the number of MSCs in UCB at different gestational ages remains unknown. Therefore, we quantified the number of UCB-MSCs at different gestational ages by flowcytometer and investigated the rate of their proliferation.

2. Materials and methods

2.1. Collection of UCB samples and multi-colour flowcytometry

A total 52 UCB samples of full-term ($n=12$; 37–40 weeks) and non-full-term ($n=40$; 28–36 weeks) were collected from healthy women (average age 24 ± 2 years) who have undergone Caesarean, with ethical approval and written informed consent in Shengjing Hospital of China Medical University, Shenyang, China. The number of MSCs in UCB was quantified by multi-colour flowcytometer (BD LSRFortessa™). In brief, three test tubes containing 100 μl UCB were prepared for each sample. Erythrocytes were first removed with FACS lysing solution (BD). CD34 PERCP (Biolegend), CD44 PE (Santa Cruz), CD45 PECY7 and CD105 FITC (Biolegend) antibodies were added to two tubes, but none to the third as the negative control. The tubes were incubated for 10 min at room temperature and centrifuged at 400–500 ***g*** for 5 min. The supernatant was discarded, and the cells were washed twice with PBS buffer. Resuspended cells in 500 μl PBS were analysed by flowcytometry using BD's analysis software.

2.2. Culture of UCB-MSCs and immunocytochemistry

Samples of 10–20 ml UCB were layered on to Lymphoprep™ (1.077 g/ml; Nycomed) for centrifugation at 850 ***g*** for 25 min. Isolated mononuclear cells, suspended in DMEM (Dulbecco's modified Eagle's medium) containing 10% fetal bovine serum, 100 units/ml penicillin, 100 mg/ml streptomycin, 2.5 μg/ml fungizone and 2 mM L-glutamine, were seeded into T75 flasks at 1×10^5 cells/cm^2 and incubated at 37°C in a humidified atmosphere with 5% CO_2/95% air. MSCs successfully cultured from each sample were selected for analysis of growth kinetics. For immunocytochemical examination, UCB-MSCs were fixed in 4% (w/v) paraformaldehyde for 24 h and washed by PBS. The cells were blocked with 5% BSA for 1 h at room temperature before being incubated with 1:100 CD45 and CD90 rabbit anti-human polyclonal antibody (Santa Cruz) overnight at 4°C. After

[1] Correspondence may be addressed to either of these authors (email pxining@yahoo.com or gangli@ort.cuhk.edu.hk).

Abbreviations: MSC, mesenchymal stem cell; UCB, umbilical cord blood.

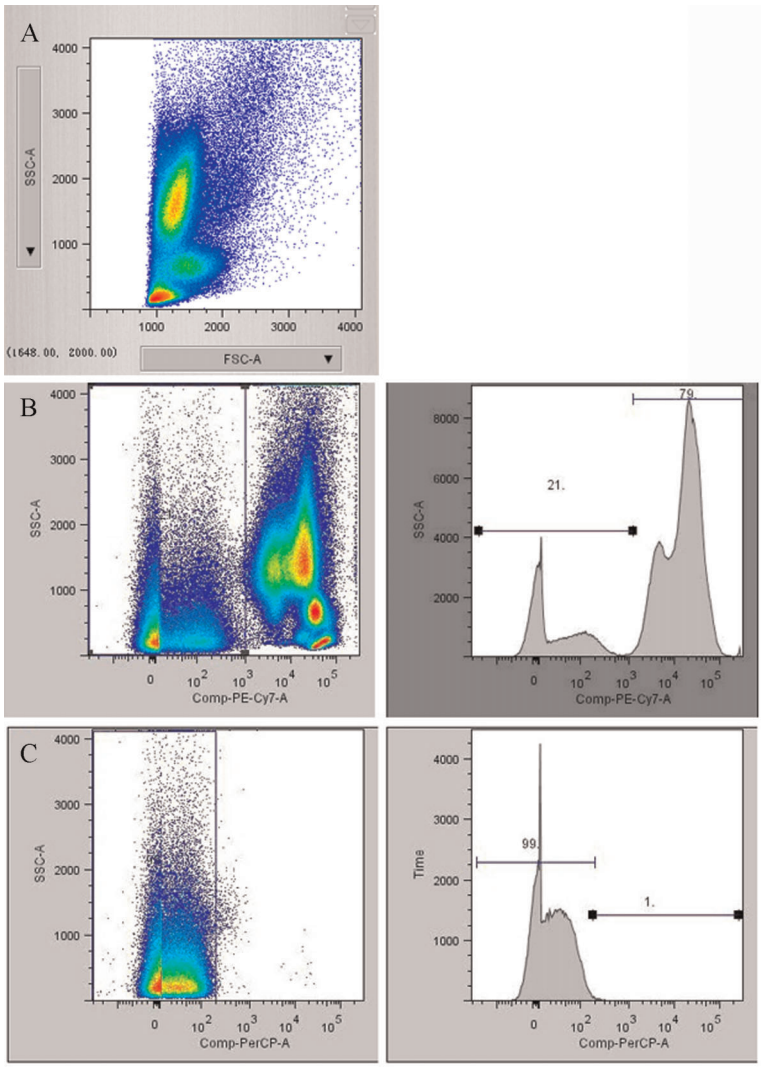

Figure 1 **Flow cytometry analysis of cord blood cell population**
(**A**) Analysis of lysed whole cord blood using FSC/SSC plot. There were three populations: granulocytes (up), monocytes (middle) and lymphocytes (down). (**B**) CD45-PECY7 fluorescence intensity distribution of lysed whole cord blood. The left panel is shown as a plot, whereas the right panel is a histogram. Up to 21% cells of total lysed whole cord blood were CD45−. (**C**) CD34-PERCP fluorescence intensity distribution of CD45− cells. CD45− cells were selected for analysis of expression of CD34. The left panel shows a plot, whereas right panel is a histogram. Over 21% cells of total lysed whole cord blood were also CD34−.

washing in PBS, cells were incubated with PE (phycoerythrin)-labelled goat-anti-rabbit antibody at room temperature for 1 h in the dark. After washing in PBS, the cells were examined under a fluorescence microscope.

3. Results

Lysed whole cord blood was examined for cell characteristics, cell size (FSC) being plotted against the presence of cytoplasmic granules (SSC). The cells were divided into three distinct types, lymphocytes, monocytes and granulocytes (Figure 1A). Positive or negative expression of surface CD markers (CD44+/CD105+/CD34−/CD45) was defined by the intensity of their fluorescence. CD45− cells accounted for 21% of the total (Figure 1B). C34 PERCP expression estimation in CD45− cells indicated that 99% were also negative for CD34 (Figure 1C). CD34−/CD45− cells were examined for their expression of CD105 and CD44. CD34−/CD45− cells can be classified into four zones according to their CD44-PE and CD105-FITC fluorescence intensity (Figure 2A). Q2 zone contained CD44+/CD105+/CD34−/CD45− cells, which represented MSCs. All samples were sorted from the different groups at intervals of 3 weeks of gestation. The average number of MSCs calculated based on every 10^6 nucleated cells was examined. MSCs gradually decreased as gestation proceeded (Figure 2B).

Full-term and non-full-term cord blood MSCs were cultured *in vitro* to explore their character. In the 12 full-term UCB samples, only one gave rise to fibroblastic and adherent cells after 96 h in

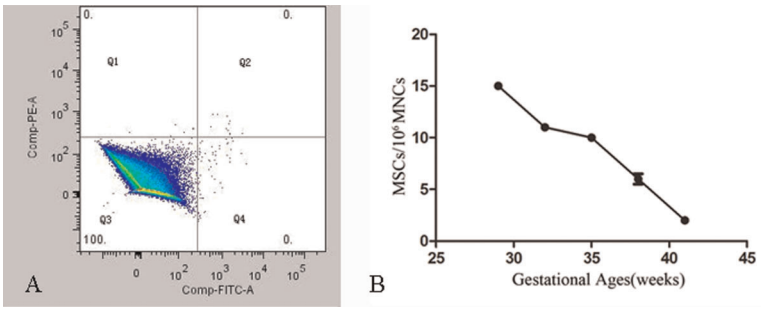

Figure 2 **MSC numbers in cord blood decline with increasing weeks of gestation**
(**A**) CD44-PE and CD105-FITC fluorescence intensity distribution of CD45−/CD34− cells. They were subsequently selected for CD44 and CD105 expression. Q2 zone represents the number of CD44+ and CD105+ cells. (**B**) CD44+/CD105+/CD34−/CD45− per 10^6 MNCs shows decline with increasing gestation.

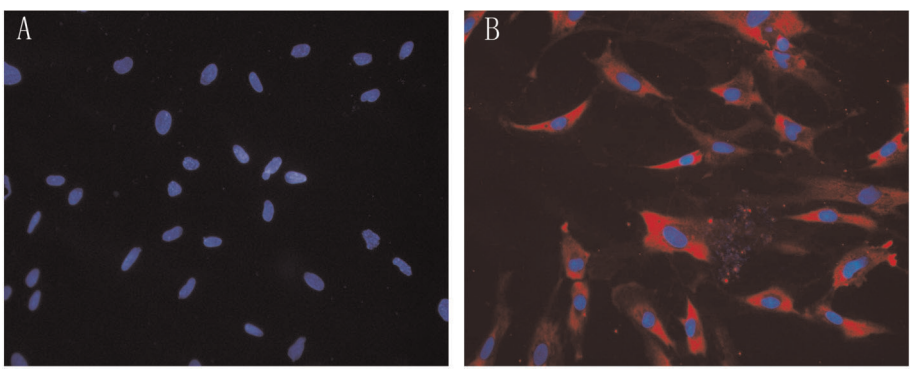

Figure 3 **Immunohistochemical staining of CD markers confirms the phenotype of UCB-MSCs**
UCB-MSCs that are CD45− (**A**), but CD90+ (**B**). MSCs cultured from UCB were fixed and stained with PE conjugated antibodies, the nuclei being counterstained with DAPI × 400.

culture compared with one in four of the non-full-term UCB within 72 h. Success of the MSCs culture rate of full-term and non-full-term UCB was 8.3 and 25.0% respectively. Successfully cultured MSCs cells were CD45− and CD90+ in the immunofluorescence assay (Figures 3A and 3B), typical characteristic surface markers of MSCs. The average number of CD44+/CD105+/CD34−/CD45− (MSCs) cells was 6.2±0.2 cells and 10.3±0.2 cells per 10^6 mononuclear cells in the full-term UCB and the non-full-term group respectively. Non-full-term MSCs proliferated significantly faster than full-term MSCs ($P<0.01$; Figure 4).

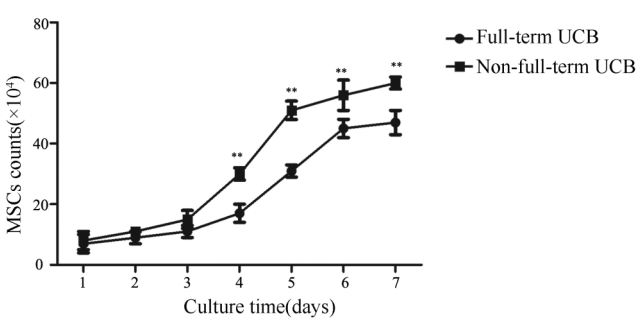

Figure 4 **Comparison of the growth kinetics of full-term and non-full-term UCB-MSCs**
UCB-MSCs were plated at 10^4 cells/cm^2. Duplicate cultures were harvested each day for 7 days, and the number of adherent cells determined. Statistically difference (**$P<0.01$) was found after day 4 between full-term and non-full-term UCB-MSCs. Results are expressed as means±S.D.

4. Discussion

MSCs isolated from multiple tissues have different frequency and expansion potentials, but their basic characteristics are similar (Kern et al., 2006). UCB provides a new resource for these young progenitor cells, but the low frequency of isolation has been a hurdle in their clinical applications. This low yield suggested that MSCs are sparse in UCB, the previous methods estimating MSCs by counting the number of clones per 10^6 cells mononuclear cells plated (Campagnoli et al., 2001), giving an inaccurate reflection of the actual quantity of MSCs because some may have been lost during the isolation and others being non-adherent.

Flowcytometery has been used to quantify the number of MSCs from bone marrow and UCB, using a combination of CD markers (Martins et al., 2009). We used a single-tube multi-colour fluorescence staining technique to analyse expression of CD44, CD105, CD34 and CD45 in the mononuclear cells at different

stages of gestation, as defined above. The average number from 30-week fetuses was 15 per 10^6 mononuclear cells, declining to 8 per 10^6 at 42 weeks [MSCs are extremely rare (mostly undetectable) in normal adult peripheral blood (Zvaifler et al., 2000; Khosla et al., 2006)]. Campagnoli et al. (2001) found similar results after comparing colony formation of first, second and third trimester UCB samples, as was also true of bone marrow, where the number of MSCs decline with aging. Bone marrow MSCs from older adults show accelerated senescence and limited expansion potential. Successfully cultured MSCs are typically spindle-shaped, and they also expressed CD90, but not CD45. The very limited number of MSCs in UCB seems to have been responsible for the difficulty in their isolation. The successful rate of MSCs isolation was significantly (2–3-fold) higher in non-full-term (near term or pre-term) UCB samples than in full-term UCB samples, and the cell proliferation rate of MSCs of pre-term UCB was also significantly higher. The data suggest the beneficial use of pre-term UCB samples for MSCs isolation and other clinical applications.

Author contribution

Lan-Lan Ma collected and/or assembled data, and was involved in data analysis and interpretation. Fan-Biao Meng, conceived and designed the study and was involved in data analysis and interpretation, they also wrote the paper. Ping Shi conceived and designed the study. Gang Li conceived and designed the study and approved the final paper. Xining Pang conceived and designed the study, and obtained the financial support.

Funding

This work was supported the China Shenyang Science and Technology Program [grant numbers 090063, F10-222-4-00 and F11-262-9-01] and National Key Basic Research and Development of China [973 Program, 2012CB518100].

References

Campagnoli C, Roberts LAG, Kumar S, Bennett PR, Bellantuono I, Fisk N. Identification of mesenchymal stem/progenitor cells in human first trimester fetal blood, liver and bone marrow. Blood 2001;98:2396–402.

Kern S, Eichler H, Stoeve J, Klüter H, Bieback K. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. Stem Cells 2006;24:1294–301.

Khosla S, Eghbali-Fatourechi GZ. Circulating cells with osteogenic potential. Ann NY Acad Sci 2006;1068:489–970.

Manca M F, Zwart I, Beo J, Palasingham R, Jen LS, Navarrete R et al. Characterization of mesenchymal stromal cells derived from full term umbilical cord blood. Cytotherapy 2008;10:54–68.

Martins AA, Paiva A, Morgado JM. Quantification and immunophenotypic characterization of bone marrow and umbilical cord blood mesenchymal stem cells by multicolor flow cytometry. Transplant Proc 2009;41:943–6.

Stenderup K, Justuesen J, Clausen C, Kassem M. Aging is associated with decreased maximal life span and accelerated senescence of bone marrow stromal cells. Bone 2003; 33:919–26

Zvaifler NJ, Marinova-Mutafchieva L, Adams G, Edwards CJ, Moss J, Burger JA et al. Mesenchymal precursor cells in the blood of normal individuals. Arthritis Res 2000;2:47788.

Received 20 March 2011/23 November 2011; accepted 10 January 2012

Published as Immediate Publication 10 January 2012, doi 10.1042/CBI20110173