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SHORT COMMUNICATION

Role of CD271 enrichment in the isolation of mesenchymal stromal cells from umbilical cord blood

Armin Attar[1,2,3]*, Arash Ghalyanchi Langeroudi[4,5], Attyieh Vassaghi[1,2], Iman Ahrari[1,2], Mohsen Khosravi Maharlooei[1,2] and Ahmad Monabati[6]

1 Student Research Committee, Shiraz University of Medical Sciences, Shiraz, Iran
2 Cell and Molecular Medicine Club, Shiraz University of Medical Sciences, Shiraz, Iran
3 Department of Cardiovascular Medicine, Shiraz University of Medical Sciences, Shiraz, Iran
4 Tasnim Biotechnology Research Center (TBRC), AJA University of Medical Sciences, Tehran, Iran
5 Faculty of Veterinary Medicine, Department of Microbiology, University of Tehran, Tehran, Iran
6 Department of Pathology, Shiraz University of Medical Sciences, Shiraz, Iran

Abstract

Isolation of mesenchymal stromal cells (MSCs) from the umbilical cord blood (UCB) has a success rate of 25% and is frequently contaminated by osteoclast-like cells (OLCs). CD271 is a well-known marker for the enrichment of bone marrow (BM) MSCs. We have assessed the effect of CD271 isolation on the isolation rate of MSCs from UCB. Twenty-one samples of UCB were collected. Ten samples of UCB and five of BM underwent CD271 isolation using magnetic activated cell sorting. The other 11 UCB samples were used as the control. The isolated cells were cultured and MSC isolation was confirmed with respect to morphology, flow cytometry, adipogenic and osteogenic differentiation potentials. CD271-positive UCB cells did not show outgrowth despite 54.5% MSCs isolation in the non-enriched portion. No OLC was noted in the CD271-enriched group, but 66% of the non-enriched samples were contaminated. All the CD271-positive BM cells formed MSC colonies. Although the per cent of CD271+ cells showed no difference between BM-mononuclear cells (MNCs) and UCB-MNCs, the haematopoietic marker, CD45, was found in a higher percentage of CD271-positive UCB-MNCs. The results of our study indicate that, although CD271 is a valuable marker for enrichment of MSCs from BM, it does not contribute to isolation of MSCs from UCB. In this source, most of the CD271+ cells are from haematopoietic origin, and possibly the process of isolation may eliminate the very low frequent MSCs and the isolation therefore fails.

Keywords: CD271; mesenchymal stem cell; mesenchymal stromal cell; osteoclast like cell; umbilical cord blood

Introduction

Multipotent stromal cells (MSCs) are suitable cells for clinical applications (Maharlooei et al., 2011), but there are some limitations in their clinical application. Proliferation, differentiation ability and therapeutic potential of MSCs are reduced with age (Bieback et al., 2004). Primarily, the umbilical cord blood (UCB) was purposed to serve young haematopoietic progenitor MSCs that are highly proliferative (Lee et al., 2004). But equivocal results appeared in that some have denied the presence of MSCs in UCB (Mareschi et al., 2001; Wexler et al., 2003; Yu et al., 2004), but others claimed that under the proper conditions they reached an isolation rate up to 60% (Bieback et al., 2004). Some parameters including sample volume (at least 33 mL) and using specialised media (such as MesenCult) improve the isolation of MSCs (Bieback et al., 2004; Vassaghi et al., 2013). Furthermore, cultures from UCB units are frequently contaminated with a population of heterogeneous cells, primarily named as the osteoclast-like cells (OLCs), with cytoplasmic extensions and occasionally multiple nuclei (Erices et al., 2000; Musina et al., 2007). Kuçi et al. (2010) showed that enrichment of CD271 can isolate bone marrow (BM) MSCs with a higher proliferative capacity in

***Corresponding author:** e-mail: attarar@sums.ac.ir

Abbreviations: APC, allophycocyanin; BM, bone marrow; FITC, fluorescein isothiocyanate; LNGFR, low-affinity nerve growth factor receptor; MACS, magnetic activated cell sorting; MNC, mononuclear cells; MSC, multipotent stromal cell; OLCs, osteoclast-like cells; PE, phycoerythrin; PerCP, peridinin chlorophyll protein; UCB, umbilical cord blood

comparison to MSCs isolated by plastic adherence. We have examined the effect of CD271-positive selection on the success rate of MSC isolation from UCB.

Materials and methods

Isolation of mononuclear cells from cord blood and bone marrow

A total of 21 samples of 47 ± 2 mL of UCB were collected after term normal vaginal delivery and were processed no later than 8 h after collection. All the women gave their written informed consent before enrolling in this study. This study is approved by the local Ethics Committee and conformed to the declaration of Helsinki. Two to three millilitres of five BM samples, taken as positive controls to assess proper isolation of CD271+ cells, were obtained from patients that underwent diagnostic BM aspiration after taking informed written consent. Cultures from samples with normal histopathology results were included in this study. The BM or UCB units were loaded on LymphoSep (1.077 g/mL, Biosera, Ringmer, UK) and centrifuged at 450 g for 25 min. The mononuclear cells (MNC) were isolated from the buffy-coat layer. MNCs from 11 UCB units were cultured directly as the control group. MNCs from the other 10 UCB units and BM samples underwent CD271 isolation for culturing or flow cytometric analysis.

Magnetic cell sorting of MNCs based on CD271

The isolated MNCs primarily underwent dead cell removal using Dead Cell Removal kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) to decrease the chance of non-specific cell bindings of antibodies. Magnetic activated cell sorting (MACS) was used to separate the CD271+ cells. MNCs were labelled with phycoerythrin (PE)-conjugated CD271 antibody (Miltenyi Biotec). The target cells were labelled with the anti-PE microbeads (Miltenyi Biotec), and the isolated cells were used for the next steps.

Culturing of the isolated cells

MNCs from the control group and CD271-positive cells from BM or UCB plated within in MesenCult basal medium supplemented with 10% mesenchymal stem cell stimulatory supplements (StemCell Technologies, Vancouver, Canada) supplemented with 4 mM GlutaMAX, 100 U/mL penicillin and 100 μg/mL streptomycin (both from Gibco/Invitrogen, Carlsbad, CA, USA) and cultured for 72 h at 37°C in air plus 5% CO_2 at 90% humidity. Media were changed twice weekly until the flasks became confluent or no cell growth was noted for 4 weeks.

Adipogenic and osteogenic differentiation

For adipogenic differentiation, third passage MSCs or the cells after magnetic cell sorting were cultured in MesenCult medium supplemented with 10% adipogenic stimulatory supplements (both from Stem Cell Technologies, Inc., Vancouver, BC, Canada). To check the differentiation, the cells were analysed with oil-red O staining and also underwent RT-PCR. For osteogenic differentiation, the cells sorted by magnetic means were cultured in NH-osteoDiff Medium (Miltenyi Biotec). To check the differentiation, the cells were stained with alizarin red and also processed by RT-PCR.

Reverse transcription-polymerase chain reaction

RT-PCR was used to determine the expression of adipocyte and osteoblast marker genes in induced cells. To assess

Table 1 PCR primer sequences and their amplified product size.

Gene	Primer sequence	Position	Product length (bp)
β-Actin[a]	5′-ATCATGTTTGAGACCTTCAA-3′	Forward	317
	5′-CATCTCTTGCTCGAAGTCCA-3′	Reverse	
PPAR-γ2	5′-TTCTCCTAT TGACCCAGAAAGC-3′	Forward	307
	5′-CTCCACTTTGATTGCACTTTGG-3′	Reverse	
ap2	5′-GCCAGGAATTTGACGAAGTC-3′	Forward	107
	5′-TGGTTGATTTTCCATCCCAT-3′	Reverse	
Osteopontin	5′-TTCCAAGTAAGTCCAACGAAAG-3′	Forward	181
	5′-GTGACCAGTTCATCAGATTCAT-3′	Reverse	
Col 1αI	5′-AAGCCGAATTCCTGGTCT-3′	Forward	195
	5′-TCCAACGAGATCGAGATCC-3′	Reverse	

Col 1α1, pro-alpha1 chains of type I collagen; PPAR-γ2, peroxisome proliferator-activated receptor gamma, aP2, adipocyte protein 2.
[a]Beta-actin is a relatively stable cytoskeletal protein that is ubiquitously expressed and serves as an internal control.

the osteogenic differentiation, RT-PCR for detection of osteopontin and col-1α1 (pro-alpha1 chains of type I collagen), mRNA was analysed. To assess the differentiation into adipocytes, RT-PCR was used in the detection of *PPAR-γ2* (peroxisome proliferator-activated receptor gamma, transcript variant 2) and *aP2* (adipocyte protein 2). The primers sequences are shown in Table 1.

Flow cytometry

To analyse the antigen panel of MNCs from BM and UCB, $>10^6$ live cells were analysed by flow cytometry. MNCs were incubated with following anti-human antibodies: CD90-fluorescein isothiocyanate (FITC) (AbD Serotec, Kidlington, Oxford, UK), CD271-PE, MSCA1-allophycocyanin (APC) and CD45-peridinin chlorophyll protein (PerCP; all from Miltenyi Biotec).

To analyse the cell surface antigen expression on the culture-derived cells, the cells were incubated with following anti-human antibodies: CD90-FITC, CD105-PE (AbD Serotec), CD14-PE, CD34-FITC, CD166-PE, CD44-FITC, CD73-PE (BD Biosciences, San Jose, CA, USA), CD45-PerCP and CD271-PE (Miltenyi Biotec). Flow cytometric analysis involved a FACS Calibur instrument (BD Biosciences).

Statistical analysis

SPSS version 19.0 for windows (IBM, US) was used for statistical analysis using the using Kruskall–Wallis and Fisher's exact test. $P < 0.05$ was considered statistically significant.

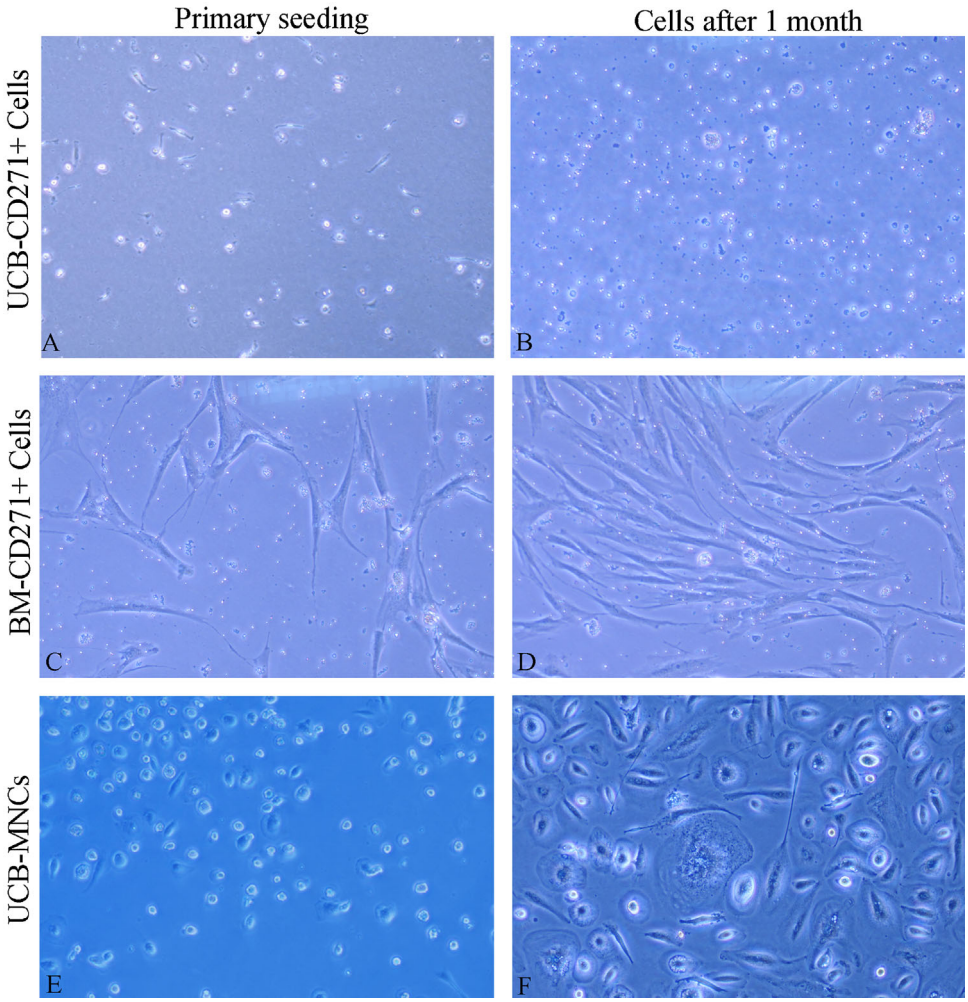

Figure 1 **(A) UCB-CD271+ cells after the initial seeding showed a round variable size morphology.** (B) All UCB-CD271+ cells after 1 month died. (C) BM-CD271+ cells after the initial seeding formed fusiform fibroblast-like appearance. (D) BM-CD271+ cells after 1 month confluented the flask with fibroblast-like cells. (E) UCB-MNCs after the initial seeding consisted of round and fusiform variable size morphology cells. (F) UCB-MNCs after 1 month showed a mixed growth of OLCs with oval/round or elongated appearance accompanied by visible borders and some showing cytoplasmic extensions or occasionally multiple nuclei and MSCs with fusiform fibroblast like-appearance (UCB, umbilical cord blood; BM, bone marrow; MNC, mononuclear cell; OLC, osteoclast like cell).

Table 2 Results from cultured samples.

Variable	Percentage of successful isolation
UCB units underwent CD271 isolation	0/10 (0%)
UCB units without CD271 isolation	6/11 (54.5%)
Total UCB units	6/21 (28.6%)
BM units underwent CD271 isolation	5/5 (100%)
Variable	**% of OLCs presence**
UCB units underwent CD271 isolation	0/10 (0%)
UCB units without CD271 isolation	7/11 (66%)
Total UCB units	7/21 (33.3%)
BM units underwent CD271 isolation	0/5 (0%)

UCB, umbilical cord blood; BM, bone marrow; OLC, osteoclast like cell.

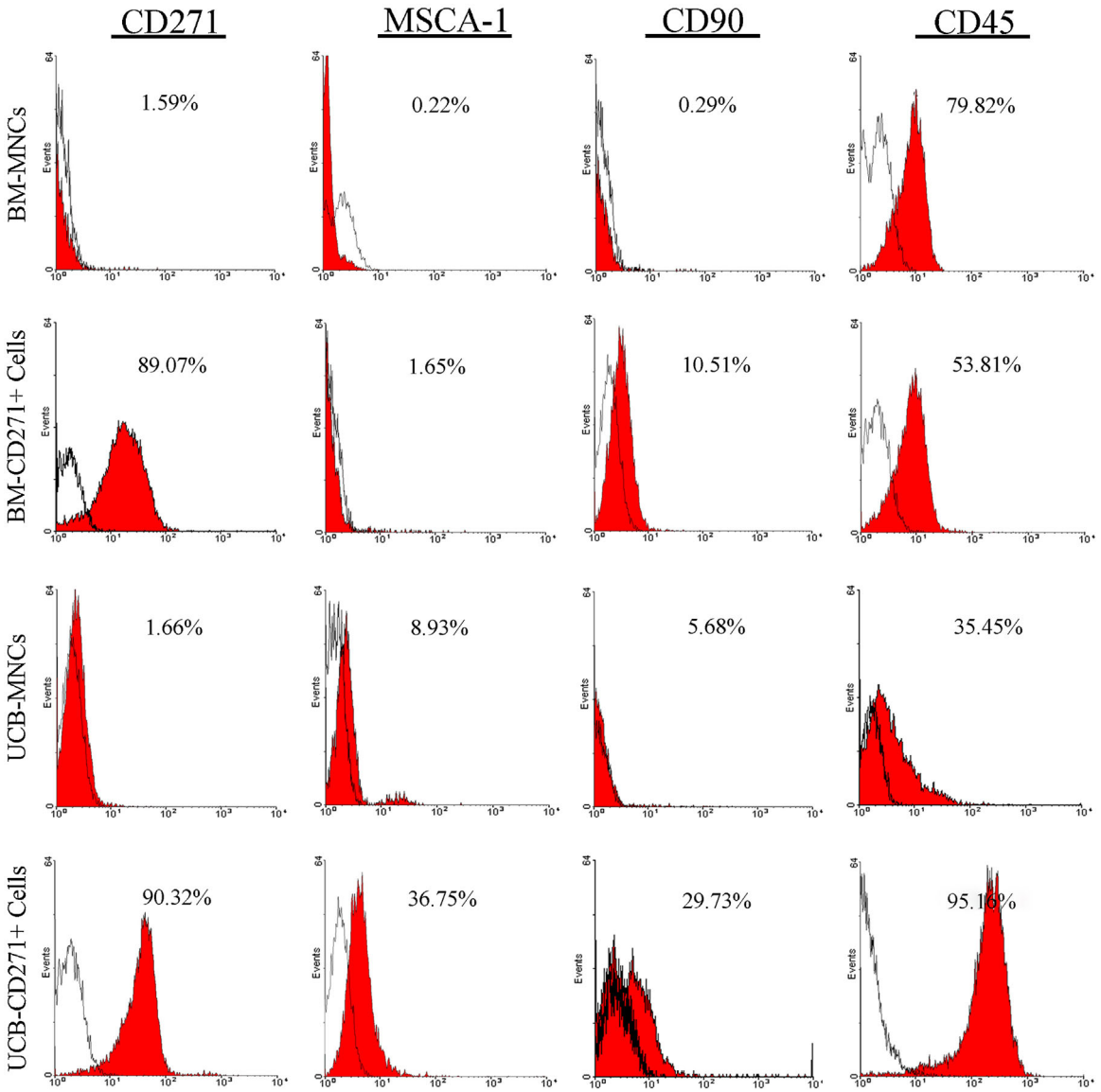

Figure 2 **Results from flow cytometry.** Percent of cell surface markers such as CD45, CD90 and MSCA1 within target populations are compared before and after CD271 isolation from BM-MNCs and UCB-MNCs (UCB, umbilical cord blood; BM, bone marrow, MNC, mononuclear cell, MSCA1, mesenchymal stem cell antigen 1).

Results

Culture characteristics

UCB-CD271+ isolated cells after the primary seeding showed non-uniform rounded appearance with variable sizes (Figure 1A). After 4 weeks, most of the cells died in the samples and no colony formation was found (Figure 1B). In contrast, BM-CD271+ cultured cells formed typical fusiform fibroblast-like cells in all five samples ($P < 0.001$; Figure 1C), which formed homogenous colonies that become confluent within 15–21 days (Figure 1D). In the control group, the primary seeded MNCs showed the same morphology as above (Figure 1E), but different results were noticed as time passed. Of 11 control samples, six samples contained fibroblast-like cells, five of which were contaminated by OLCs (Figure 1F). Two other flasks contained only OLCs (Table 2). This shows the higher possibility of MSC isolation from UCB-MNCs than UCB CD271-positive cells ($P = 0.012$).

Differentiation assay

Differentiated adipocytes had vacuoles that stained with oil-red O. These cells expressed PPAR-γ2 and aP2 mRNAs, as shown by RT-PCR. Mineralisation of the cell differentiated to osteoblasts was documented by alizarin red staining. The differentiated cells typically expressed osteopontin and col-1α1.

Flow cytometric results

The dead cell analysis with propidium iodide showed that >92% of cells were alive after removing the dead cells. The % of CD271+ cells showed no difference between BM-MNCs and UCB-MNCs ($P = 0.9$). The haematopoietic marker CD45 was found in a higher % of CD271-positive cells. Data from analysis of BM and UCB-MNCs before and after CD271 isolation are gievn in Figure 2. Flow cytometric analysis of the fibroblast-like cells harvested from UCB-derived cultures and CD271+ BM derived culture showed that the cells were positive for mesenchymal markers such as CD73, CD105, CD44, CD166, CD90, and negative for the haematopoietic antigens CD45, CD34 and CD14. Although CD271+ BM derived cells uniformly expressed CD271, only a partial expression of CD271 was noted in the UCB derived cells (Table 3).

Table 3 Results from flow cytometry of the cells resulted from cultures that could successfully form MSCs colonies.

	CD271	CD90	CD166	CD105	CD34	CD14	CD45	CD73	CD44
Cultured CD271+ BM-MSCs	79.85 ± 5.67	89.28 ± 10.31	99.88 ± 0.1	97.66 ± 1.93	0.91 ± 0.62	0.47 ± 0.12	1 ± 0.47	98.68 ± 1.24	100
Cultured UCB-MSC	53.4 ± 6.87	92.28 ± 3.27	97.68 ± 0.23	98.97 ± 0.73	2.24 ± 0.75	1.69 ± 0.33	0.98 ± 0.38	92.23 ± 2.14	99.78 ± 0.12

Data are shown as the mean % of analysed cells ± standard deviation.
UCB, umbilical cord blood; BM, bone marrow; MNC, mononuclear cell; MSCA1, mesenchymal stem cell antigen 1.

Discussion

Magnetic cell sorting and culture techniques were used isolate mesenchymal stem cells from UCB and characterise them by

flow cytometry and differentiation assays, taking the BM MSCs as the controls. We took into consideration previously mentioned parameters proposed for increasing the isolation rates of MSC from UCB and had a success rate of 54.5% in isolation of MSCs from non-enriched UCB-MNCs, similar to the results of Bieback et al. (2004). BM CD271+ cells from all the samples, taken as the positive control, could successfully form colonies with appropriate cell surface antigen marker panel and differentiation potential. On the other hand, no colony outgrowth was noted in the CD271+ UCB counterparts ($P < 0.001$). This shows the higher possibility of MSC isolation from unsorted UCB-MNCs than UCB CD271-positive cells ($P = 0.012$). Our results are in agreement with previous data indicating that CD271 is a useful marker for isolation of MSCs from BM (Kuçi et al., 2010). However, that this cannot be extended to UCB; the profile of CD271+ cells in BM and UCB differs and the haematopoietic markers (e.g. CD45) gave a higher % of CD271-positive cells. This clarifies that most of CD271+ cells in the UCB are from haematopoietic origin rather than MSCs. We encountered the problem of OLCs presence in the cultures of unsorted UCB-MNCs as others had found (Bieback et al., 2004; Wagner et al., 2005; Musina et al., 2007), but no OLC was seen in the cultures from CD271+ cells.

Our results show that CD271 enrichment does not help isolation of MSCs from sources like UCB, in which almost all of the CD271+ cells are from haematopoietic origin and the frequency of MSCs is so low that possibly during the processes of cell isolation most of them are lost, such that isolation fails.

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Conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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