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Parameters that influence the isolation of multipotent mesenchymal stromal cells from human umbilical cord blood

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BACKGROUND AND OBJECTIVES: Umbilical cord blood is an important source of stem cells. However, isolating multipotent mesenchymal stromal cells (MSCs) from umbilical cord blood presents methodological challenges. We compared the effectiveness of six approaches to improve the success rate of MSC isolation and proliferation from umbilical cord blood.

METHODS: Thirty umbilical cord blood units underwent investigation. In 10 samples, MNCs from each sample were divided into four groups to test the effect of negative immunodepletion (NI) alone (group A); NI plus basic fibroblastic growth factor (bFGF) supplementation together (group B); bFGF supplementation alone (group C); and culture with neither NI nor bFGF (group D). The cells of each group were isolated from 10 mL of umbilical cord blood. For investigating the effect of sample volume (group E) and MesenCult Proliferation Kits (group F), cells were isolated from 45 ± 2 ml. MSCs were identified on the basis of morphological, flow cytometric and differentiation potential characteristics.

RESULTS: In groups of A–D, one week after the initial seeding, the cells showed a rounded appearance, and in the fourth week, many of them died. MSCs outgrowth was seen in 40% of the samples from group F, and this yield was further enhanced to 60% in cultures done with the MesenCult Proliferation Kit (group F). The fibroblast-like cells expanded rapidly and showed features of MSCs.

CONCLUSION: Sample volume was the parameter that showed the greatest influence on the isolation yield of MSCs from umbilical cord blood. This could be further enhanced by adding the MesenCult Proliferation Kit.

Multipotent mesenchymal stromal cells (MSCs), previously known as mesenchymal stem cells,[1] are a group of multipotent clonogenic cells originally found mainly in the bone marrow. These cells play a major role in hematopoiesis as the supportive stroma for hematopoietic stem cell growth and differentiation in vivo. They can be derived from adipose tissue, dental pulp and umbilical cord blood (UCB) in addition to bone marrow. Their most notable characteristic is their capability for multi-lineage differentiation into cells such as osteoblasts, adipocytes, chondrocytes, cardiomyocytes, endothelial cells,[2] hepatocytes,[3] and myoblasts[4] in vitro.

Their simple ex vivo expansion, immunomodulatory properties[5] and good differentiation characteristics make these cells interesting candidates for clinical cell therapies, and their use in clinical trials has been approved in many countries.[2,6] However, there are some limitations on their clinical use. As an instance, it is well known that the proliferation and differentiation capacities as well as the therapeutic potential of MSC may decrease with age.[7] Therefore UCB, which has long been regarded as a source of young hematopoietic progenitors suitable for transplantation, was believed to be an easily accessible source of young and highly proliferative MSCs.[8] However, research soon raised questions about this

assumption. Some studies denied the presence of MSC in UCB,[9–11] whereas others have claimed that under suitable conditions an isolation rate up to 60% can be achieved.[7] Generally, an enrichment rate of around 30% is the widely reported result in most studies.[12–18]

The most important cause of the limited success of MSC isolation is the low frequency of these cells in UCB. Culture techniques have shown the presence of only 1–2 MSC clones per 10^8 mononuclear cells of UCB, whereas in fresh bone marrow, MSC may account for 1–100 per million nucleated cells.[18,19] This fact led Bieback et al. to propose sample volume (at least 33 mL) as a critical parameter for the isolation of these cells.[7] In contrast, Rebelatto et al. reported that sample volume did not have any influence on the success rate of MSC isolation.[17]

Earlier research reported improvements in MSCs isolation by negative immunodepletion (NI) of $CD3^+$, $CD14^+$, $CD19^+$, $CD38^+$, $CD66b^+$ and glycophorin A^+ cells with commercial kits.[8] Some studies found that supplementation of the culture medium with the powerful mitogen basic fibroblastic growth factor (bFGF) enhanced the proliferation, differentiation and survival of stem cells.[8,20] However, other attempts with NI were unsuccessful.[17] Bieback et al. found that use of a specialized medium (such as MesenCult Proliferation Kit, StemCell Technologies, Vancouver, Canada) can improve the isolation rate of MSC from UCB. They have also suggested that UCB units should not be processed later than 15 h after collection.[7] Javed et al. noticed that MSCs were rarely identified in 37–40 weeks of gestational age in UCB but frequently isolated from 24 to 28 weeks gestational aged UCB.[21]

The final problem of working with UCB units is the contamination of cultures with a population of heterogeneous cells with cytoplasmic extensions and occasionally multiple nuclei[12,19] in 80–90% of culture plates.[7] These cells were primarily named as the osteoclast-like cells (OLCs) by Erices et al.[12]

Due to controversies regarding the optimization of culture conditions, as well as the high costs for some kits and supplements, we designed the present study to investigate the effectiveness of different culture media supplementation in improving the proliferation and isolation of MSC from UCB.

METHODS

Collection of cord blood units

A total of 30 samples of umbilical cord blood were collected after full term normal vaginal delivery. After delivery and during detachment of the placenta from the uterus, blood was allowed to drain from the end of the cord into 50 mL-tubes containing 500 µL 1 mM EDTA as the anticoagulant. Each sample consisted of a mean of 47 ± 2 mL UCB, which was processed no later than 8 h after collection.

Collection of bone marrow units

A volume of 2–3 mL of bone marrow, anticoagulated with heparin, was obtained from patients who underwent diagnostic bone marrow aspiration after informed written consent. Cultures from samples with normal results were included in this study.

Isolation and culture of MNC from UCB

We used six different culture methods. In 10 samples, MNCs from each sample were divided into four groups to test the effect of NI alone (group A); NI plus bFGF supplementation together (group B); bFGF supplementation alone (group C); and culture with neither NI nor bFGF (group D as the control). For culturing each of the groups A–D, a volume of 10 mL blood from the same UC unit was used. Negative immunodepletion of $CD3^+$, $CD14^+$, $CD19^+$, $CD38^+$, $CD66b^+$, and glycophorin A^+ cells was done with RosetteSep kits (StemCell Technologies, Vancouver, Canada) according to the manufacturer's instructions. After that, blood was loaded on LymphoSep (1.077 g/mL, Biosera, Ringmer, UK) and centrifuged at 450 g for 25 min. Mononuclear cells (MNC) were then isolated from the buffy coat layer. In groups C and D, cells were isolated only by gradient density centrifugation with LymphoSep as in groups A and B.

Isolated MNC from each group were plated in uncoated T25 culture flasks (Orange Scientific). Groups B and C cells were seeded in α-MEM (Invitrogen) supplemented with 20% FBS (Gibco), 10 ng/mL bFGF (Invitrogen), 4 mM GlutaMAX (Invitrogen), 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen). Cells from groups A and D were cultured in the same media without bFGF.

The other 20 samples were used to test the influence of large sample volume (group E) and MesenCult Proliferation Kit supplementation in large volumes (group F). In groups E and F, all MNCs were used in the experiment. Group F cells were cultured with the MesenCult Proliferation Kit (StemCell Technologies, Vancouver, Canada) supplemented with 4 mM GlutaMAX (Invitrogen), 1000 U penicillin and 1000 µg streptomycin (Invitrogen) according to the manufacturer's instructions, and group E cells were cultured in α-MEM (Invitrogen) supplemented with

20% FBS (Gibco), 4 mM GlutaMAX (Invitrogen), 100 U/mlL penicillin and 100 μg/mL streptomycin (Invitrogen).

All flasks were incubated at 37 °C in 95% humidified air containing 5% CO_2. Media of all flasks were changed four days after the initial seeding and non-adherent cells were removed by medium exchange. Thereafter all media were changed twice a week until the flasks became 80% confluent or until no cell outgrowth was noted after two months. Cell isolation and expansion were recorded every other day.

Cells from flasks that reached 80% confluency were harvested by 0.25% trypsin–EDTA (Invitrogen) and were replated 1:3 in the expansion medium.

Isolation and culture of MNC from BM

The bone marrow (BM) was loaded on LymphoSep (1.077 g/mL, Biosera, Ringmer, UK) and centrifuged at 450*g* for 25 min. Mononuclear cells (MNC) were then isolated from the buffy coat layer. Isolated MNCs were plated in uncoated T25 culture flasks (Orange Scientific) in α-MEM (Invitrogen) supplemented with 10% FBS (Gibco), 4 mM GlutaMAX (Invitrogen), 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen).

Flow cytometry

To analyze cell surface antigen expression, cells from the third passage were harvested by 0.25% trypsin–EDTA. Trypsin was neutralized with medium containing FBS, and the isolated cells were washed twice with PBS. Then the cells were incubated for 30 min in the dark with the following anti-human antibodies: CD90-fluorescein isothiocyanate (FITC), CD14-phycoerythrin (PE), CD34-FITC, CD166-PE, CD45-FITC, CD44-FITC, CD105-PE (Serotec, UK), and CD73-PE (Becton Dickinson, USA). Mouse IgG1-PE and IgG2a-FITC (Serotec) were used as the isotype controls. Cells were analyzed in a BD FACS-Calibur instrument.

Osteogenic differentiation

For osteogenic differentiation, MSCs from the third passage were harvested by 0.25% trypsin–EDTA. The cells were cultured in NH-OsteoDiff Medium (Miltenyi Biotec, Germany) at a density of 3×10^4 cells/mL in 2-chamber culture slides (Becton Dickinson) for four weeks according to the manufacturer's instructions, and the medium was changed twice per week. To verify differentiation after morphological changes were observed, the cells were analyzed by alizarin red staining. Briefly, the cells were washed once with PBS and fixed in methanol for 10 min. They were stained with the solution of 0.1 M alizarin red (Sigma–Aldrich) in a 25% aqueous solution of ammonia for 24 h, and then washed once with distilled water.

Adipogenic differentiation

For adipogenic differentiation, cells from the third passage were harvested as reported above and cultured in MesenCult medium (StemCell Technologies) supplemented with 10% Adipogenic Stimulatory Supplements (StemCell Technologies) at a density of 1.5×10^4 cells/mL in 2-chamber culture slides according to the manufacturer's instructions. The cells were cultured for three weeks and half of the medium was changed each time the medium turned yellow. After the cells showed appropriate morphological changes, they were analyzed with Oil-red O staining. Briefly, the cells were fixed in 4% formalin containing 1% calcium chloride for 1 h. The cells were then stained with Oil-red O solution for 10–15 min and counterstained with 70% ethanol for 1 min, and washed with distilled water. The solution was made with 0.05 M powder (Sigma–Aldrich) in 99% isopropranol diluted 3:2 with distilled water.

Statistical analysis

The data were analyzed with SPSS statistical software. Chi-squared tests were used to assess the significance of the difference between parameters. $P < 0.05$ was considered statistically significant.

Ethical consideration

The UCB was collected after legal consent.

RESULTS

Culture characteristics

With NI, approximately one third of the cells were depleted in groups A and B. In groups C and D, the cell population was heterogeneous in shape and size. One week after the initial seeding, the cells in all four groups showed a rounded shape. By the fourth week, most of the cells were dead in all four groups and no CFU-F units were noted.

Only one or two fibroblast-like colonies were seen in four out of 10 flasks of group E cultures, apart from a few rounded cells. These fibroblast-like cells interestingly formed homogenous colonies (Figure 1B) that were confluent within 15 days. The formations of fibroblast-like colonies were enhanced in group F (Figure 1A), where six of 10 flasks developed colonies with the morphological appearance of mesenchymal cells (Table 1).

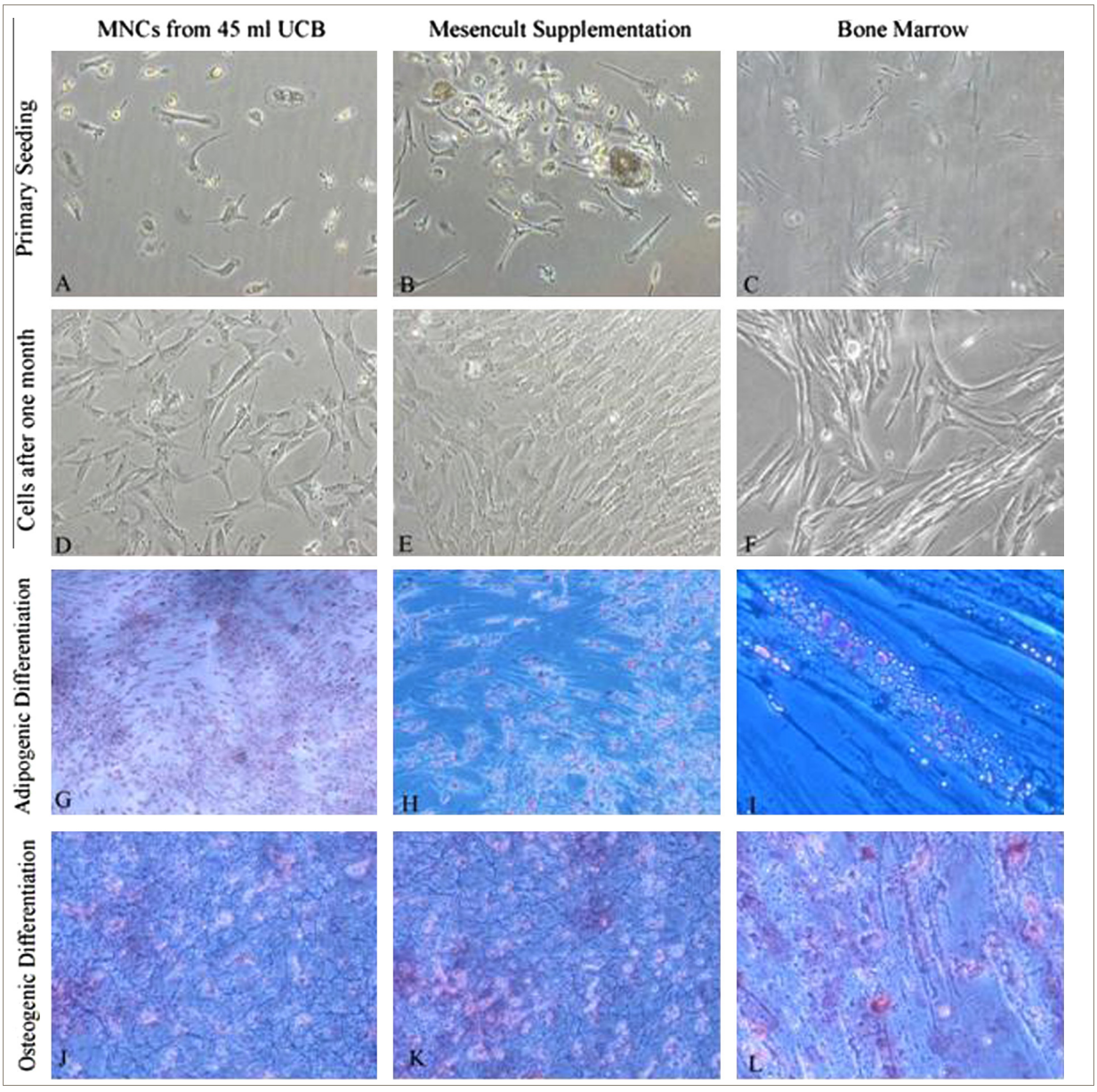

Figure 1. Cells one week after initial seeding in (A) group E, (B) group F and (C) bone marrow MNCs. Spindle shaped confluent fibroblast like cells noted one month after initial seeding within the flasks from (D) group E, (E) group F and (F) bone marrow. Fat vacuoles stained by Oil red O 21 days after cells were cultured in adipogenic media in (G) group E, (H) group F and (I) bone marrow MSCs. As it is seen UCB-MSCs form smaller vacuole than BM-MSCs. Osteoblasts stained with Alizarin red four weeks after culturing in osteogenic media from (J) group E cells, (K) group F cells and (L) bone marrow MSCs.

In two flasks from group E and five flasks from group F, oval/round or elongated cells with visible borders were seen. Some cells showed cytoplasmic extensions and occasional multiple nuclei. These cells were considered as OLCs (Figure 2A). Interestingly, OLCs could become confluent within two months (Figure 2B). These cells were detached hardly by trypsin. After passaging and subculturing, they adhered and proliferated again, although their proliferation was limited (Figure 2C). In flasks where both MSCs and OLCs were present, the OLCs were occasionally surrounded by a monolayer of small spindle-shaped mononuclear cells which were probably MSC (Figure 2D). Group F cultures more frequently contained OLCs than cultures of group E (Table 2). We observed OLCs in cultures that used sample volumes of less than 45 mL, and NI was not able to eliminate these cells (Figure 2A).

Table 1. Yield of stem cell isolation from umbilical cord blood under different cell culture conditions.

Culture group	Variable	UCB volume	UCB unit	Stem cell growth
A	NI only	10 mL	10	0
B	NI and bFGF supplementation	10 mL	10	0
C	bFGF supplementation	10 mL	10	0
D	Control(1)	10 mL	10	0
E	Large sample volume	50 mL	10	6 of 10
F	MesenCult Proliferation Kit	50 mL	10	4 of 10
Total			30	10 of 30

Abbreviations: UCB, umbilical cord blood; NI, negative immunodepletion; bFGF, basic fibroblast growth factor.

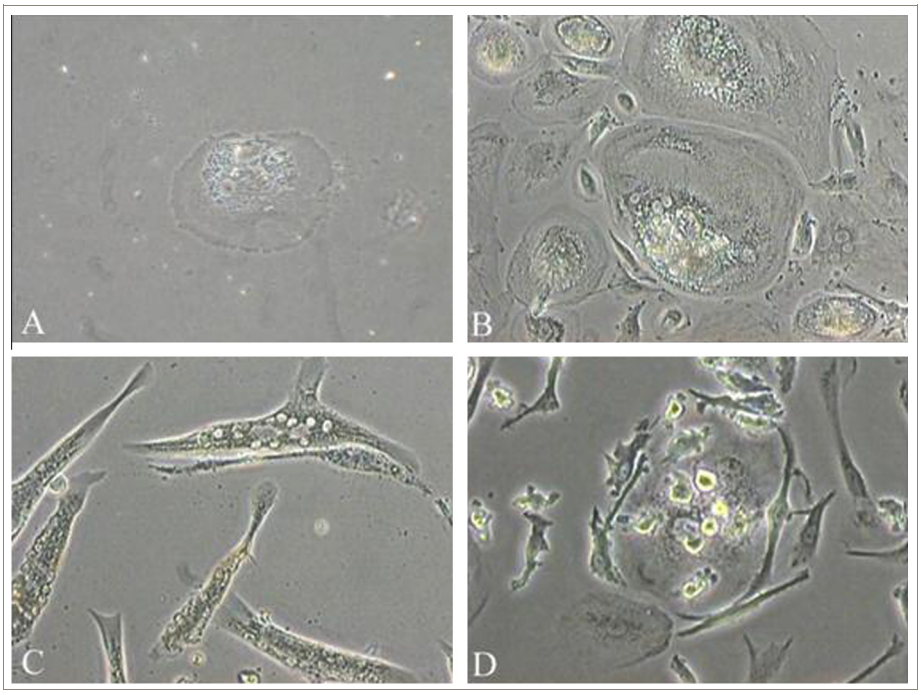

Figure 2. (A) An osteoclast like cell (OLC) in group B culture (B) Confluent OLCs in group E after two months. (C) OLCs 3 days after passage were large and spindle-shaped, multinucleated, and showed cytoplasmic extensions, making them clearly distinguishable from mesenchymal stem cells. (D) A multinuclear OLC surrounded by spindle-shaped cells.

Overall, we isolated mesenchymal stem cells from 10 samples within 30 umbilical cord units (success rate: 33.3%). None was from groups A–D. The isolation yield in group E was 40% which enhanced to 60% with MesenCult Proliferation Kit administration (Table 1).

Two days after primary cultivation of BM-MNCs, adherent spindle-shaped cells were detected (Figure 1C). Expanded cells showed homogenous morphology and these fibroblast-like cells formed homogenous colonies that confluented within 20 days (Figure 1F).

Flow cytometric analysis

Flow cytometric analysis of the cells harvested from UCB-derived cultures showed that the cells were positive for mesenchymal markers such as CD73 (98.68 ± 1.24%), CD105 (97.66 ± 1.93%), CD44 (100%), CD166 (99.88 ± 0.1%) and CD90 (89.28 ± 10.31%); and negative for hematopoietic antigens such as CD45 (1 ± 0.87%), CD34 (0.91 ± 0.62%) and CD14 (0.47 ± 0.21%) (Figure 3).

Table 2. Yield of osteoclast-like cell growth from umbilical cord blood under different cell culture conditions.

Culture group	Variable	OLC growth	Mesenchymal growth	Mixed growth of OLCs and MSCs
A	NI only	1 of 10	0 of 10	0 of 10
B	NI and bFGF supplementation	0 of 10	0 of 10	0 of 10
C	bFGF supplementation	0 of 10	0 of 10	0 of 10
D	Control(1)	0 of 10	0 of 10	0 of 10
E	Large sample volume	5 of 10	6 of 10	4 of 10
F	MesenCult Proliferation Kit	2 of 10	4 of 10	2 of 10
Total		8 of 30	10 of 30	6 of 30

Abbreviations: UCB, umbilical cord blood; NI, negative immunodepletion; bFGF, basic fibroblast growth factor.

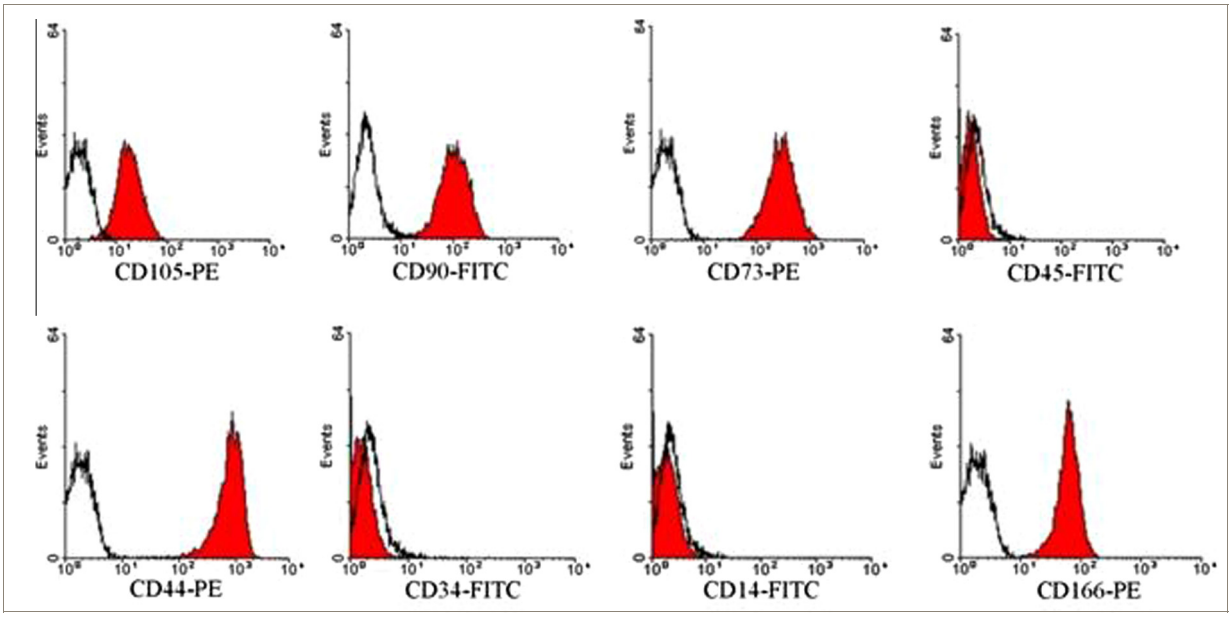

Figure 3. Immune phenotype of umbilical cord blood-derived fibroblast-like colony-forming cells.

Differentiation assay

Adipogenic differentiation was documented by both morphological and staining criteria. One week after seeding in the adipogenic medium, the cells showed small isolated vacuoles that increased in number and size with time (Figure 1E). These vacuoles did not become as large as vacuoles in bone marrow-derived MSC cultures but all were stained by Oil red O (Figure 1G–I).

The earliest evidence of MSC differentiation to osteoblasts was matrix depositions around the cells in the second week after seeding. Full differentiation to osteoblasts lasted four weeks. Mineralization was documented by alizarin red staining (Figure 1J–L).

Statistics

The yield difference between high volume sample and low volume sample was significant ($P < 0.05$) but the statistic analysis between MesenCult group (group F) and control 2 (group E) did not show any significance (P value > 0.05). P value of OLC growth between MesenCult group (group F) and control 2 (group E) was higher than 0.05.

DISCUSSION

Mesenchymal stem cells are multipotent cells that can generate adipogenic, osteogenic, chondrogenic, myogenic and even endothelial cell lineages.[2,7] Because of their potential, increasing interest is focusing on the

isolation of MSC capable of differentiating into mesodermal lineages for transplantation and tissue engineering. Cells isolated for cell therapy should fulfill the criteria proposed by the International Society for Cellular Therapy[22] to be considered as MSCs. One of the most important sources of both hematopoietic and mesenchymal stem cells is UCB. However, the success rates of MSC isolation and proliferation from UCB are low. Some reports[9–11] claim that MSC isolation and expansion from UCB are unsuccessful, but our results show that UCB CFU-Fs are true MSCs and all our findings provide sufficient data confirming the fact that UCB does contain MSCs.

Like Wagner et al., we faced the crucial problem of the low number of MSC in UCB in comparison to bone marrow, which makes their isolation difficult.[14] Researchers have managed to isolate these cells from UCB, albeit with low success rates. From a total of 644 UCB units processed for MSC isolation in different studies, only 167 outgrowths have been reported (26% success rate).[12–17] This figure is similar to our success rate of 33.3%. These results show that further improvements are needed in the isolation of UCB MSC.

Our data showed that the RosetteSep commercial kit did not affect the purification of MSCs from UCB. Our findings cannot be compared with the findings of Lee et al. regarding the influence of NI and bFGF supplementation, since they did not mention the sample volume from which they isolated MNC.[8] In large volume samples NI may facilitate MSC isolation. However, we do not recommend NI as it was not effective in the present set of experiments. Rebelatto et al. also found this technique to have no effect on MSC yield.[17] Furthermore, NI significantly increases the costs of isolation.

We noticed that bFGF could not increase the isolation rate of MSCs. Furthermore, previous reports showed that bFGF can increase the expression of HLA class I, thus reinforcing the immune reaction in allogenic transplantation and decreasing the engraftment of transplanted cells.[20,23]

While Rebelatto et al. claimed that sample volume did not correlate with MSC isolation,[17] others have proposed that sample volume (at least 33 mL[7] or 45 mL[19]) is a critical parameter. In groups E and F we used a sample volume of 45 mL of UCB. We found that sample volume was the most significant parameter in influencing the success rate of UCB MSC isolation ($P < 0.05$). In addition, we found that the success rate of MSC isolation from a large volume of UCB units was increased by the MesenCult Proliferation Kit as much as 60% ($P < 0.05$).

We encountered the problem of OLC presence as other researchers did.[7,12,19] Bieback et al. reported the presence of monocytic cells that fused to form OLCs in about 80–90% of their culture plates—a much higher yield of OLC than we obtained.[7] The difference between studies probably reflects differences in the definition or description of these cells, which would have led to different interpretations of the results of cell cultures. In this research, we considered only cells with the same low proliferation capacity and cytoplasmic extensions as OLC. The presence of these cells in cultures is a significant problem that limits the use of UCB as a source of MSC for cell therapy. Culture techniques appear unable to eradicate them, and we found that even NI of $CD3^+$, $CD14^+$, $CD19^+$, $CD38^+$, $CD66b^+$, and glycophorin A^+ cells with the RosetteSep kit did not prevent their appearance (Figure 3A). The flow cytometric analysis of OLC published by Erices et al. showed that common leukocyte antigen CD45 was uniformly expressed on these cells.[12] These authors found that osteoclast-related antigen CD51/CD61 (vitronectin receptor) was also seen on OLC, but only a few subpopulations expressed the CD14 antigen. Because CD14 is not uniformly present on OLC, the RosetteSep kit used for NI cannot eradicate these cells.

We found that although the MesenCult Proliferation Kit enhanced UCB MSC isolation, this supplement also supported the growth and adhesion of OLC. The frequency of OLC in cultures supplemented with the MesenCult Proliferation Kit was higher than other cultures but not statistically significant ($P > 0.05$).

The surface markers of UCB MSC in the present study were similar to those identified on MSC derived from bone marrow,[17] i.e., CD73, CD90, CD105, CD166 and CD44. In both studies, these cells were negative for the hematopoietic cell antigens CD14, CD34 and CD45. In contrast to the report by Bieback et al.,[7] CD105 (SH2) was expressed by 97.66 ± 1.93% of our UCB-MSCs, a proportion similar to that of bone marrow-derived MSC.[18]

Our data contradict the findings of Kern et al. that UCB-derived MSC cannot differentiate to adipose tissue.[15] We found that UCB-derived MSC differentiated into adipose tissue after about 35 days of culturing in an adipogenic medium. But in contrast to bone marrow-derived MSC that developed large, round lipid vacuoles, adipocyte differentiated UCB-MSCs developed numerous small lipid vacuoles. The same findings were noted by Rebelatto et al.[13] and Karahuseyinoglu et al.[24]

CONCLUSION

In general, our results showed that the number of MSC in UCB is low in comparison to other common sources; therefore further efforts are needed to improve the success rate of isolation. We increased the success rate of isolation up to 60% by using a larger sample volume and applying the MesenCult Proliferation Kit. According to our data, the use of the RosetteSep kit did not improve the isolation of these scarce cells. We showed that UCB-derived MSC can differentiate to adipose and osteogenic tissue, but this requires longer culture periods in special media. Obtaining pure MSCs from UCB is made difficult by the appearance of OLC in the cultures. We suggest that CD45 depletion from UCB-MNCs containing at least 10^8 cells may be an alternative for OLC-free isolation of multipotent mesenchymal stromal cells from umbilical cord blood.

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