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Original Article

Critical Parameters for the Isolation of Mesenchymal Stem Cells from Umbilical Cord Blood

KAREN BIEBACK, SUSANNE KERN, HARALD KLÜTER, HERMANN EICHLER

Institute of Transfusion Medicine and Immunology, German Red Cross Blood Service of Baden-Württemberg-Hessen, Germany; University of Heidelberg, Faculty of Clinical Medicine Mannheim, Germany

Key Words. Mesenchymal stem cells · Umbilical cord blood · Isolation · Multilineage differentiation

ABSTRACT

Evidence has emerged that mesenchymal stem cells (MSCs) represent a promising population for supporting new clinical concepts in cellular therapy. However, attempts to isolate MSCs from umbilical cord blood (UCB) of full-term deliveries have previously either failed or been characterized by a low yield. We investigated whether cells with MSC characteristics and multilineage differentiation potential can be cultivated from UCB of healthy newborns and whether yields might be maximized by optimal culture conditions or by defining UCB quality criteria.

Using optimized isolation and culture conditions, in up to 63% of 59 low-volume UCB units, cells showing a characteristic mesenchymal morphology and immune phenotype (MSC-like cells) were isolated. These were similar to control MSCs from adult bone marrow (BM). The frequency of MSC-like cells ranged from 0 to 2.3 clones per 1×10^8 mononuclear cells (MNCs). The cell clones proliferated extensively with at least 20 population doublings within eight passages. In addition, osteogenic and chondrogenic differentiation demonstrated a multilineage capacity comparable with BM MSCs. However, in contrast to MSCs, MSC-like cells showed a reduced sensitivity to undergo adipogenic differentiation.

Crucial points to isolate MSC-like cells from UCB were a time from collection to isolation of less than 15 hours, a net volume of more than 33 ml, and an MNC count of more than 1×10^8 MNCs.

Because MSC-like cells can be isolated at high efficacy from full-term UCB donations, we regard UCB as an additional stem cell source for experimental and potentially clinical purposes. ***Stem Cells*** *2004;22:625–634*

INTRODUCTION

Mesenchymal stem cells (MSCs) comprise a rare population of multipotent progenitors capable of both supporting hematopoiesis and differentiating into at least the osteogenic, adipogenic, and chondrogenic lineages [1, 2]. These characteristics make MSCs very promising candidates to develop new cell-based therapeutic strategies, such as the treatment of mesenchymal tissue injuries or the supportive application in the context of hematopoietic stem cell (HSC) transplantation [3, 4]. Currently, bone marrow (BM) represents the main source of MSCs for both experimental and clinical studies [2–4]. As the number of MSCs and their differentiation capacity decline with age [5], their therapeutic potential might be diminished as well. Therefore, it can be argued that cells with

Correspondence: Karen Bieback, Ph.D., Institute of Transfusion Medicine and Immunology, German Red Cross Blood Service of Baden-Württemberg-Hessen, Friedrich-Ebert-Strasse 107, D-68167 Mannheim, Germany. Telephone: 49-621-3706-8216; Fax: 49-621-3706-851; e-mail: k.bieback@blutspende.de Received October 29, 2003; accepted for publication February 18, 2004.

both extensive potency of proliferation and differentiation would represent an optimal tool for future cell-based therapeutic applications.

Umbilical cord blood (UCB) has turned out to be an excellent alternative source of HSCs for clinical-scale allogeneic transplantation [6]. Essential preclinical studies proved a higher percentage of $CD34^+CD38^-$ cells in UCB compared with BM, suggesting that more primitive progenitors may be abundant in neonatal blood [7]. The same might apply for the presence of MSCs or progenitor cells. However, previous attempts to isolate MSCs from UCB have either failed [8–10] or have demonstrated a low frequency of mesenchymal progenitors in UCB of full-term deliveries [11, 12]. Therefore, the first goal of the present study was to verify whether cells with MSC traits can be isolated from full-term UCB units. Second, those cells were to be compared with BM-derived MSCs to assess potential similarities or differences. In addition, we intended to optimize MSC isolation protocols to increase the efficacy towards levels that are applicable for therapeutic approaches by modifying cell culture conditions as well as by defining quality criteria for UCB units.

Materials and Methods

Collection of UCB

UCB units from full-term deliveries were collected from the unborn placenta with informed consent of the mothers [13]. A bag system containing 17 ml of citrate phosphate dextrose (CPD) anticoagulant within the collection bag was used (Cord Blood Collection System, Eltest, Bonn, Germany). The units were stored at 22 ± 4°C before processing.

Isolation and Culture of Adherent Cells from UCB

To isolate mononuclear cells (MNCs), each UCB unit was diluted 1:1 with phosphate-buffered saline (PBS)/2 mM EDTA (Nexell, Baxter, Unterschleißheim, Germany, http://www. baxter.de, and Merck, Darmstadt, Germany, http://www.merck.com) and carefully loaded onto Ficoll-Hypaque solution (Amersham, Freiburg, Germany, http://www.amershambiosciences.com). After density gradient centrifugation at ×435 *g* for 30 minutes at room temperature, MNCs were removed from the interphase and washed two to three times with PBS/EDTA. Cell counts were performed using an automated cell analyzer (Cell-Dyn 3200, Abbott, Wiesbaden, Germany, http://www.abbott.de).

UCB-derived MNCs were set in culture at a density of $1 \times 10^6/cm^2$ into six-well culture plates (Falcon, Becton Dickinson, Heidelberg, Germany, http://www.bdbiosciences.com) in MSCGM medium (MSCGM BulletKit™, CellSystems, St. Katharinen, Germany, http://www.cellsystems.de). For example, a UCB unit with 1×10^8 MNCs was plated in 10 wells.

Where specified, MesenCult was used as growth-promoting medium (Stem Cell Technologies, St. Katharinen, Germany, http://www.stemcell.com). In contrast to MSCGM, which is based on Dulbecco's modified Eagle's medium (DMEM, low glucose) containing 10% fetal calf serum (FCS) from selected lots, MesenCult is based on McCoy's medium, also containing 10% FCS.

After overnight incubation at 37°C in humidified atmosphere containing 5% carbon dioxide, nonadherent cells were removed and fresh medium was added to the wells. Cultures were maintained, and remaining nonadherent cells were removed by complete exchange of culture medium every 7 days. Culture wells were screened continuously to get hold of developing colonies of adherent cells. The precursor frequency of UCB-derived adherent fibroblastoid cells was calculated by counting the number of colonies per 10^8 MNCs. UCB units, which displayed no colony formation, were also considered for calculation.

Fibroblastoid cells were recovered between days 16 through 20 after initial plating using 0.04% Trypsin/0.03% EDTA (PromoCell, Heidelberg, Germany, http://www.promocell.com). Recovered cells were replated at a density of 4,000 to 5,000 cells/cm^2 as passage 1 cells and thereafter.

The population-doubling level was calculated for each subcultivation by using the following equation: population doublings = $[\log_{10}(N_H) - \log_{10}(N_1)]/\log_{10}(2)$, where N_I is inoculum number and N_H is cell harvest number [14]. The calculated population-doubling increase was added to the population-doubling levels of the previous passages to yield the cumulative population-doubling level.

Monocyte Removal

To prevent stable adherence of monocytic cells, MNC fractions from 48 UCB units were plated in six-well plates precoated with FCS [15]. In detail, culture plates were precoated with FCS (batches S0113/1038E and S0113/892E, Biochrom, Berlin, Germany, http://www.biochrom.de) for 30 to 60 minutes at room temperature. After removing FCS, the plates were used immediately or stored at 4°C until further use. The MNCs were seeded as indicated above into the FCS-precoated plates. Nonadherent cells were harvested after overnight incubation. To calculate the effect of FCS coating on the adherence of monocytes, we assessed the recovery of monocytes by comparing the percentage of monocytes in the MNC fraction before and after plating by flow cytometry. The cells were labeled with CD14-FITC (Becton Dickinson) and CD45-PerCP (Beckman Coulter). To compare the effect of

FCS coating on the remaining adherent cells, photomicrographs were taken every week and the number of adherent cells was determined. Osteoclast-like cells and fibroblastoid cells were discriminated microscopically, and the number of wells with any of these types of cells was counted.

Collection and Isolation of Control MSCs from BM

BM served as positive control and was obtained from the femoral shaft of patients undergoing total hip replacement at the Orthopedic Department of the University Hospital Mannheim. Cells were aspirated into a 5-ml syringe containing CPD anticoagulant. In total, six specimens from female patients were obtained, with the donor age ranging from 68 to 84 years.

To isolate MSCs from BM, the aspirate was diluted 1:5 and processed as described above. In contrast to MNCs from UCB, BM-derived MNCs were cultured at a density of 1×10^6 cells/cm^2 in T75 culture flasks (Nunc, Wiesbaden, Germany, www.nunc.de), and the first change of medium was performed 3 days after initial plating. Two weeks later, at reaching 80%–90% confluence, MSCs were suspended and replated as described for the UCB-derived adherent cells.

Primary Fibroblasts as Controls

Primary normal human dermal fibroblasts (PromoCell) served as negative control in the differentiation studies. The cells were maintained in supplemented fibroblast growth medium (PromoCell).

Immune Phenotypic Analyses

Efforts to define a distinct phenotype characteristic for MSC have been confounded by the fact that these cells can express a range of cell lineage–specific antigens [16]. To analyze cell-surface expression of typical marker proteins, UCB- and BM-derived adherent cells, each at fourth passage, were labelled with the following anti-human antibodies: CD14-FITC, CD34-PE, CD73-PE (also referred to as SH3 and SH4 [2, 16]), CD90-Cy5 (Becton Dickinson), CD29-PE, CD44-FITC, CD45-PerCP, HLA–class I–FITC, HLA–class II–FITC (Beckman Coulter, Krefeld, Germany, http://www.beckman.com), CD133-PE (Miltenyi Biotech, Bergisch Gladbach, Germany, http://www.miltenyibiotec.com), CD105-FITC (SH2 [2, 16]), and CD106-PE (Immunokontakt, AMS Biotechnology, Wiesbaden, Germany, http://www.immunok.com). Mouse isotype antibodies served as respective controls (Becton Dickinson and Beckman Coulter). Ten thousand labelled cells were acquired and analyzed using a FACScan flow cytometer running CellQuest software (Becton Dickinson).

Differentiation Studies

Osteogenic Differentiation

To induce osteogenic differentiation, the cells were seeded at a density of 3.1×10^3 cells/cm^2 and cultured in eight-chamber slides (Nunc) until they reached approximately 80% confluence. Additional culture was performed in osteogenic differentiation medium supplemented with hMSC Osteogenic SingleQuots™ containing 0.1 μM dexamethasone, 10 mM β-glycerophosphate, 0.05 mM ascorbate, and 10% FCS (CellSystems) [17]. The onset of osteoblast formation was evaluated after 3 weeks by both the expression of alkaline phosphatase and by calcium accumulation. Activity of alkaline phosphatase was detected histologically, as described recently (Leukocyte Alkaline Phosphatase-Kit, Sigma Diagnostics, Taufkirchen, Germany, http://www.sigmaaldrich.com) [2]. The accumulation of mineralized calcium phosphate was assessed by von Kossa staining after the protocol from Cheng et al. [18] with few modifications. The cells were fixed for 15 minutes in 10% formalin (Sigma), and after washing they were incubated with 5% silver nitrate (Sigma) for 15 to 30 minutes. Pyrogallol 1% (Merck) and sodium thiosulfate 5% (Sigma) were used to develop and fix the signal.

Adipogenic Differentiation

The adipogenic differentiation was induced by cyclic changes of adipogenic induction and maintenance medium (CellSystems) in cells grown postconfluently in eight-chamber slides, following the protocol of Pittenger et al. [2]. The induction medium, containing 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methyl-xanthine, 10 μg/ml recombinant human (rh) insulin, 0.2 mM indomethacin, and 10% FCS, was added for 2 to 3 days to the culture chambers. It was then replaced by maintenance medium containing only rh insulin and 10% FCS. Control cells were kept in adipogenic maintenance medium. After three cycles of changing the two media, cultures were maintained for 1 additional week. The induction of adipogenic differentiation was apparent by intracellular accumulation of lipid-rich vacuoles that stained with Oil Red O (Fluka, Taufkirchen, Germany, http://www.sigmaaldrich.com). The cells were fixed with 10% formalin, washed, and stained with a working solution of 0.18% Oil Red O for 5 minutes.

Chondrogenic Differentiation

To promote chondrogenic differentiation, 2.5×10^5 cells were gently centrifuged (×150*g*, 5 minutes) in a 15-ml polypropylene tube (Greiner) to form a pellet according to the protocol of Mackay et al. [19]. Without disturbing the pellet, the cells were cultured for 4 weeks in complete chondrogenic differentiation medium (CellSystems) including 10

ng/ml TGFβ3 (Strathmann Biotec AG, Hamburg, Germany, http://www.strathmann-biotec-ag.de) by feeding twice a week. After the culture period, cryosections were analyzed by Safranin O staining. The sections were fixed with ice-cold acetone (Sigma) and stained with 0.1% aqueous Safranin O solution (Sigma). Cell nuclei were counterstained with Weigert's iron hematoxylin (Sigma).

Statistical Analysis

Data were presented as mean ± standard deviation (SD). Two-sided paired Student's t-test was used to compare the mean values of cell percentages and numbers in wells coated with FCS and in wells left uncoated. Two-sided nonpaired t-test was used to analyze the flow cytometry data. Chi-square (χ^2) tests were used to compare the frequency distributions according to differing quality parameters of UCB units. Differences were considered significant at $p < .05$. The SPSS software package was used for all statistical analyses (Chicago, http://www.spss.com).

Results

Generation of Primary Cultures from Adherent UCB Cells

In total, 59 UCB units entered this study and were analyzed regarding their capacity to generate cultures of MSCs.

After plating the MNCs, only a few cells attached to the plastic culture dishes and formed adherent cells within 3 weeks. Most of those cells were monocytes, which fused to form osteoclast-like cells [20]. These appeared in 80%–90% of the wells uncoated with FCS and reached a subconfluent condition within 3 to 4 weeks, as shown in Figure 1A.

Rare events displayed fibroblastoid cells, which expanded rapidly (MSC-like cells). In total, we obtained 29 colonies in 17 of the 59 processed UCB units. The onset of colony formation could be observed at first after 14 to 16 days. Cell confluence was reached after approximately 20 days of culture. In contrast, BM-derived MNCs formed clusters of spindle-shaped MSCs within 5 to 10 days and cell confluence after 2 to 3 weeks. But the UCB-derived MSC-like cells were of clonal origin, because at most one colony developed per well. Therefore, the mean (± SD) frequency of MSC-like cells was calculated from all tested UCB units to be 0.7 ± 0.2 clone-forming units per 1×10^8 MNCs of full-term UCB, ranging from 0 to 2.3 per 1×10^8 MNCs. In units that gave rise to MSC-like cells, the frequency was variable and ranged from 0.2 to 2.3 clones per 1×10^8 MNCs (mean, 1.1 ± 0.2).

Twenty-two UCB-derived clonal cell populations were spindle-shaped, but the other seven were more spherical (Figs. 1B–1D). However, they maintained this phenotype even in between subsequent passages (Figs. 1D–1F). Most clones could be cultured for at least eight passages and approximately 20 population doublings (Fig. 1G) (maximum up to now passage 12, with approximately 27 population doublings, $n = 3$), showing neither changed morphology nor reduced proliferation ($n = 21$). In contrast, all BM-derived MSCs displayed a change in morphology and markedly reduced proliferation already after approximately five population doublings (passages 3 through 4), as shown in Figures 1G–1I.

Characterization of MSC-Like Cells from UCB

Immune Phenotype

The expression of cell-surface antigens by flow cytometry was evaluated on 12 clones of MSC-like cells and 4 BM-derived MSC preparations, each at passage 4. Neither the cells derived from UCB nor from BM expressed the hematopoietic markers CD14, CD34 and CD45, and CD133 (Fig. 2). Similar to MSCs from BM, UCB-derived clones were strongly positive for CD29, CD44, and CD73. In addition, cells from both sources stained positive for HLA-class I and negative for HLA-class II. However, only 53.6 ± 4.3% of the UCB-derived cells expressed CD105 (also known as SH2) compared with 84.4 ± 2.8% of BM MSC ($p = .02$). In contrast, 72.4 ± 3.2% of the UCB cells stained positive for CD106 compared with 42.4 ± 6.5% of BM MSC ($p = .04$). In addition, although most UCB-derived and BM-derived cells expressed CD90 (95.4% and 99.9%, respectively), there was a significant difference in the intensity of antigen expression. The MSC-like cells displayed a mean fluorescence intensity of 416.4 ± 186.5, whereas BM MSCs showed a significantly higher expression with 2193.9 ± 254.8 ($p = .03$).

Differentiation Potential

The differentiation potential of all 29 UCB-derived clones was compared with BM MSCs. Cells from passage 1 or 2 were cultured under conditions that are favorable for an osteogenic, adipogenic, or chondrogenic differentiation, respectively.

First, osteogenic differentiation was induced in all BM MSC preparations and all MSC-like cell clones but not in fibroblasts (not shown), as shown by increased activity of alkaline phosphatase (Figs. 3A–3D) and enhanced mineralization defined by von Kossa staining (Figs. 3E–3H). This capacity was retained in all analyzed UCB clones ($n = 8$, passages 5 and 7).

Second, the adipogenic induction was apparent in MSCs from BM by the cellular accumulation of lipid-rich vacuoles that stained with Oil Red O (Fig. 3L). However, the fibroblasts (not shown) and, most strikingly, all 29 MSC-like cell clones never generated adipocytes under standard differenti-

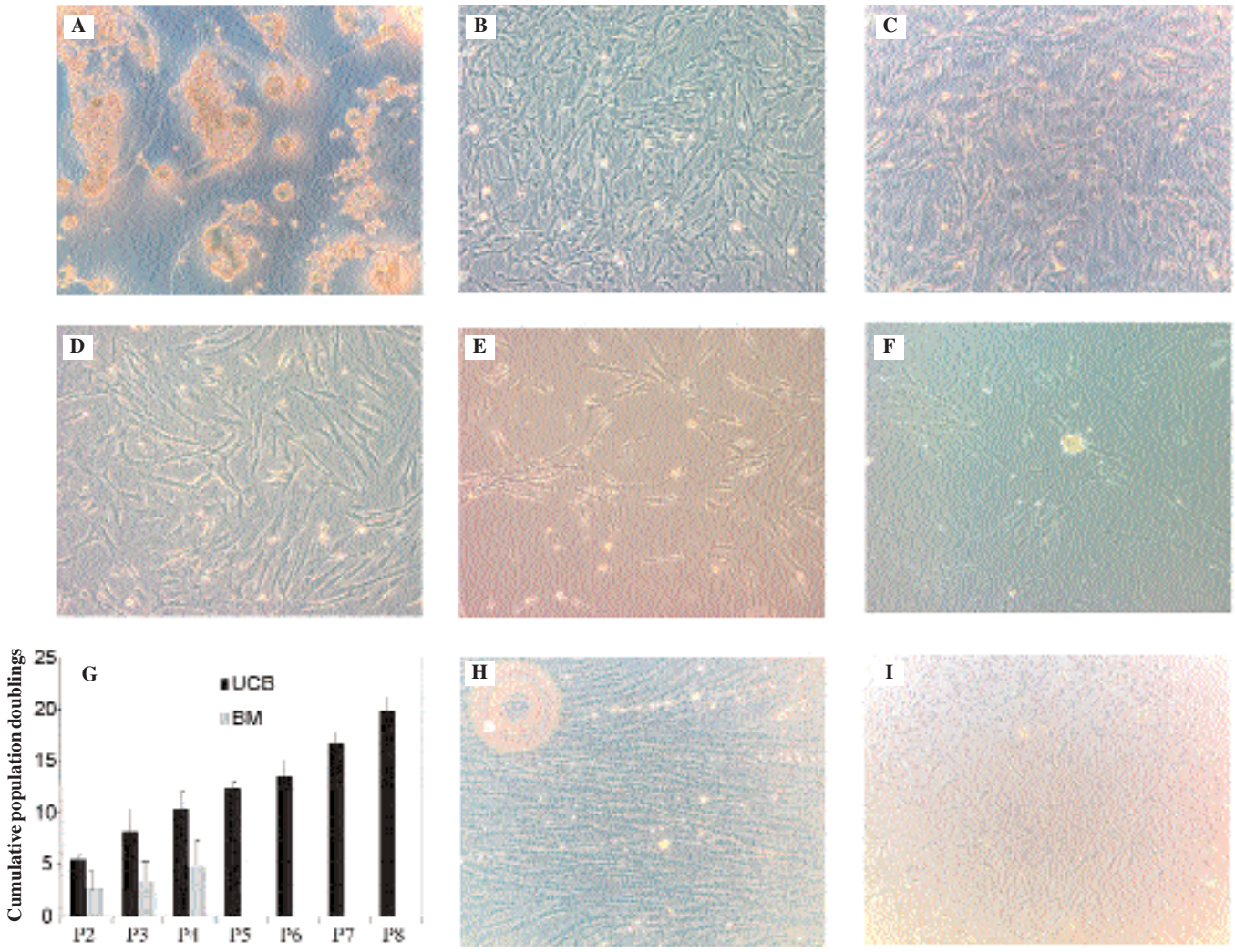

Figure 1. Morphology of UCB-derived adherent cells displaying an osteoclast-like or fibroblastoid morphology. **(A)**: Morphology of UCB-derived osteoclast-like cells in primary culture at day 16. UCB-derived fibroblastoid cells were identified as distinct colonies at days 14 through 16 after initial plating. They displayed either a consistent spindle-shaped **(B, D)** or a more spherical, less spindle-shaped morphology **(C)**. The clone shown in **(D)** maintained a homogenous phenotype until passage 8 or 19.3 population doublings. (**D**): The colony at passage 0, day 16 before split. **(F, G)**: The same clone at passage 3, day 37 (4 days after split, approximately eight population doublings) and passage 8, day 90 (4 days after split, 19.3 population doublings). BM-derived MSCs are shown as controls in **(H)** and **(I)**, with **(H)** representing cells at passage 0, reaching a confluent stage at day 14. The MSCs depicted in **(I)** present an altered morphology and a stop in proliferation already at passage 4 (day 31, 7 days after split, 4.5 population doublings). These are representative examples of 29 clones of UCB-derived fibroblastoid cells and six processed BM aspirates, shown at ×100 magnification. **(G)**: Mean values of the cumulative population doublings, determined at each subcultivation, with UCB-derived cells shown in black (n = 12) and BM-derived MSCs shown in gray (n = 5). Abbreviations: BM, bone marrow; MSC, mesenchymal stem cell; UCB, umbilical cord blood.

ation conditions. Adipogenic differentiation could solely be induced in MSC-like cells cultured continuously in adipogenic induction medium for at least 5 weeks (Fig. 3J).

Finally, in chondrogenic differentiation assays, MSC-like cells, BM MSC, and fibroblasts consolidated within 1 day, forming aggregates that dislodged to float freely in the suspension culture. Cryosections of the aggregates stained with Safranin O showed a condensed structure with chondrocyte-like lacunae (Figs. 3M and 3N, fibroblasts not shown). Also, UCB-derived MSC-like cells at later passages maintained the ability to form cartilage (n = 8).

Optimization of Culture Conditions for the Isolation of MSC-Like Cells

Initially, the frequency of MSC-like cells and the percentage of UCB units generating MSC-like cells was quite low, with one of eight processed UCB units (12.5%). Thus, we intended to improve the efficacy at first by modulating the culture con-

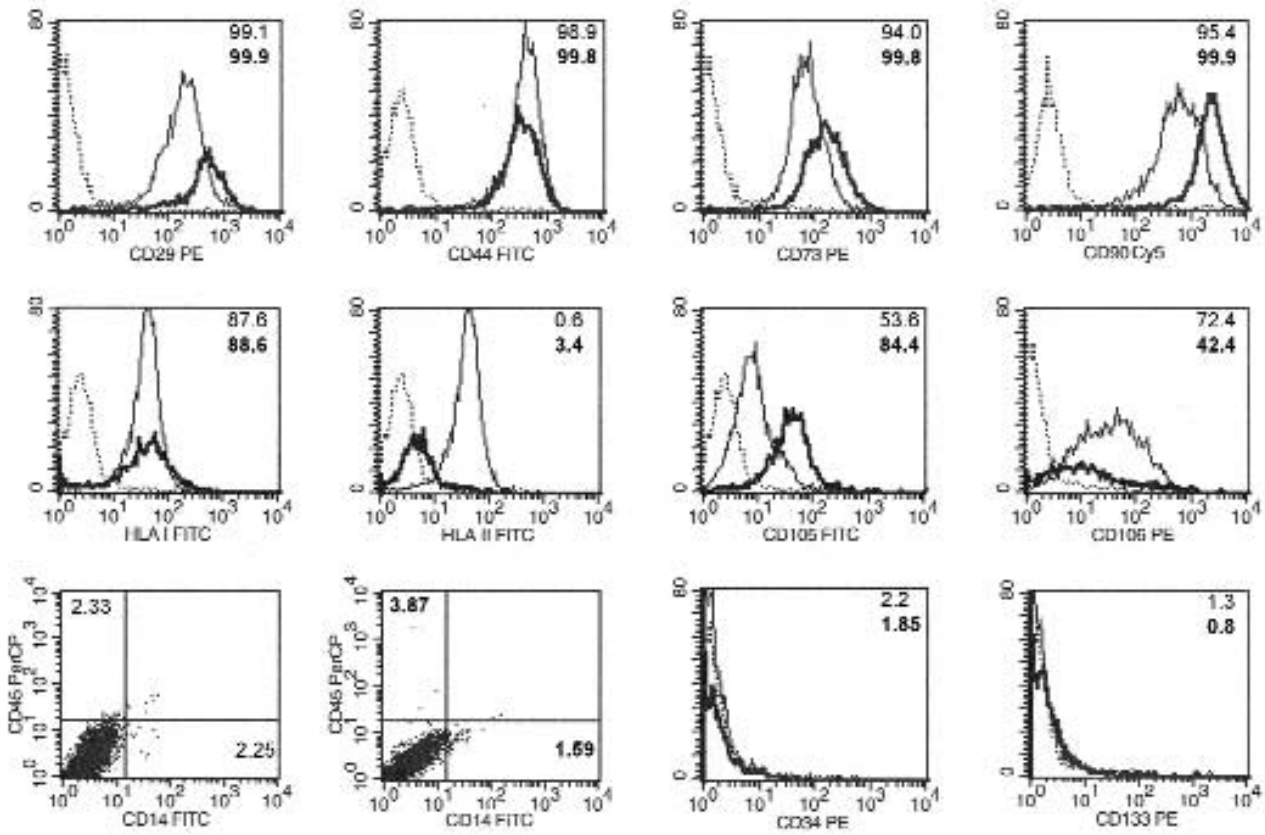

Figure 2. Immune phenotype of MSC-like cells from UCB and BM MSCs. Fibroblastoid cells from UCB and BM at passage 4 were trypsinized, labeled with antibodies against the indicated antigens, and analyzed by flow cytometry. The respective isotype control is shown as a dotted line. A representative example of 12 UCB clones ( ) and 4 BM harvests ( ) is shown. Values represent the mean percentage of all assessed cells positively stained by the respective antibodies in the flow cytometry analyses. Abbreviations: BM, bone marrow; MSC, mesenchymal stem cell; UCB, umbilical cord blood.

ditions. Because no fibroblastoid cells could be visualized in wells showing osteoclast-like cells, our first approach was to prevent the adherence of monocytes as osteoclast precursors. To dispose the monocytes, we cultured the MNC in FCS-coated wells and removed the nonadherent cells after overnight adherence. Analyzing six UCB units in parallel by plating one half of the MNC fraction in FCS-precoated wells and the other half in uncoated wells, we observed a significantly higher percentage of monocytes harvested in the nonadherent fraction after seeding MNCs in FCS-coated wells (Table 1). This indicated that fewer monocytes adhered to the coated plates than to the uncoated plates. In addition, significantly fewer adherent cells and osteoclast-like cells were achieved in the wells precoated with FCS. In consequence, we received four colonies of MSC-like cells from three units plated in FCS-precoated wells compared with two colonies from two units seeded in uncoated wells (Table 1). Therefore, although the impact of FCS coating on displaying MSCs was not significant in those six units, we proceeded using precoated wells for the isolation and gained 12 units with 22 MSC-like cell clones out of 48 UCB units.

In a second approach, we tested different commercially available media known to promote the growth of MSCs from BM. Using MSCGM, based on DMEM, 29 colonies of MSC-like cells could be generated from 53 UCB units. Using MesenCult, based on McCoys, we succeeded in isolating MSCs from BM ($n = 4$) but not from UCB ($n = 6$; $p = .04$).

Criteria for UCB Units for Efficient Isolation of MSC-Like Cells

Even after adjusting the culture conditions to optimal levels, we were only able to isolate MSC-like cells from one third of the processed UCB units (Table 2).

To establish criteria to predict an efficient isolation, we analyzed various quality aspects of the processed UCB units. Because it has been shown that cord blood from preterm

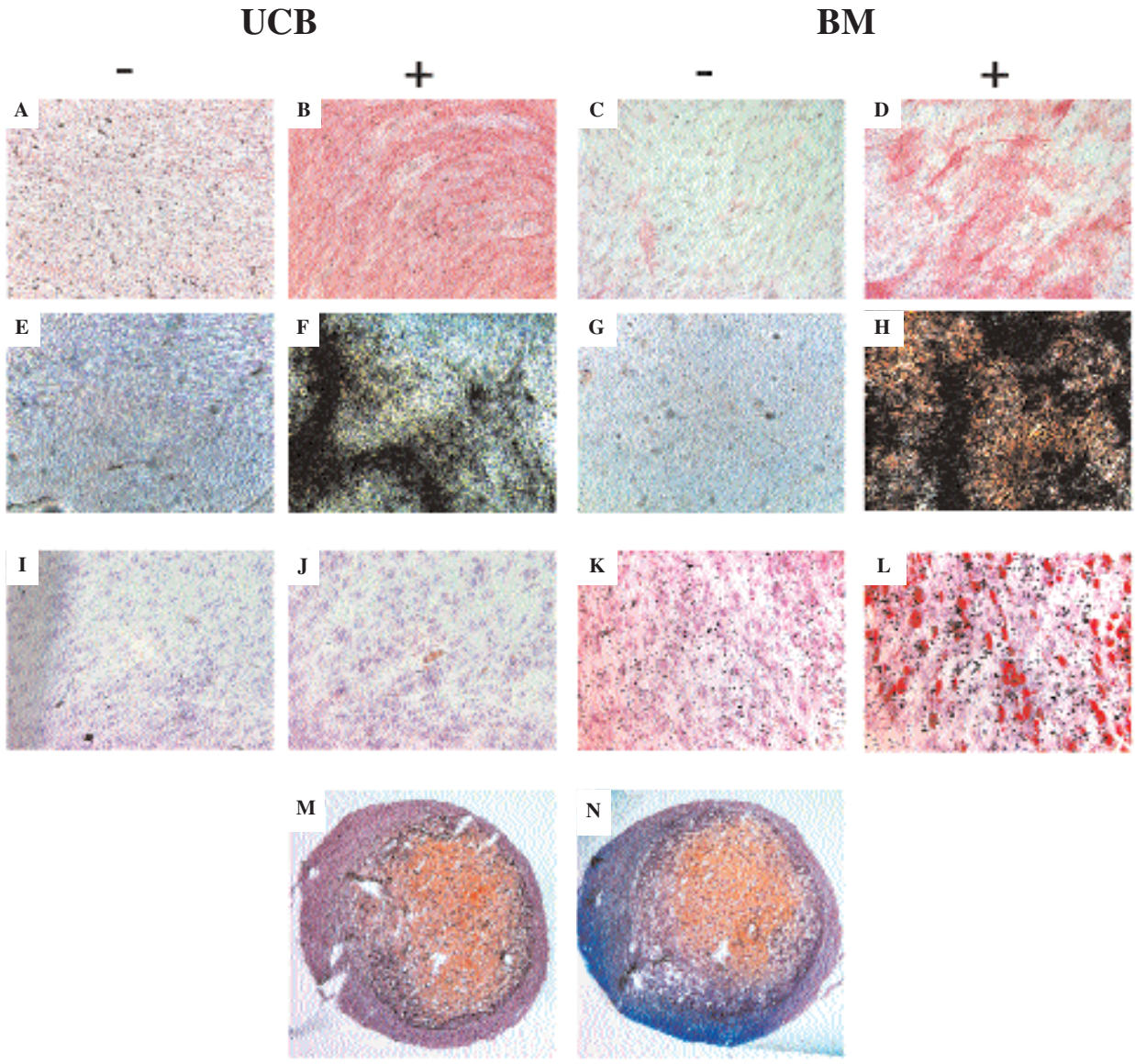

Figure 3. Differentiation capacity of MSC-like cells from UCB and MSCs from BM. Cultured cells from UCB-derived clones and BM harvests were exposed in vitro to differentiation medium to induce osteogenic, adipogenic, and chondrogenic differentiation, respectively. UCB-derived fibroblastoid cells were shown to differentiate appropriately to the osteogenic lineage by enhancement of alkaline phosphatase activity **(B)** and calcium mineralization detected by von Kossa stain **(F)** [**(A)** and **(E)** show noninduced controls]. Correspondingly, the BM MSCs used as a control are shown in **(C)**, **(D)**, **(G)**, and **(H)** (magnification ×100). Adipocytes could solely be detected by Oil Red O stain in the adipogenic cultures of UCB MSC-like cells after intense induction for at least 5 weeks [**(I)**: not induced; **(J)**: induced]. In contrast, BM MSC-derived adipocytes could easily be induced by cyclic changes of adipogenic induction and maintenance medium for 3 weeks. [**(K)**: not induced; **(L)**: induced; magnification ×200). Chondrogenesis was shown by Safranin O staining in cryosections from both the UCB-derived clonal cells in **(M)** and the BM MSC in **(N)** (magnification ×100). A representative example of 29 UCB clones (five for adipogenic differentiation) and six BM harvests at passage 1 is shown. Abbreviations: BM, bone marrow; MSC, mesenchymal stem cell; UCB, umbilical cord blood.

deliveries can contain MSCs [11], we tried to correlate the presence of MSCs with either the gestational age or the mode of delivery. The gestational age ranged from 37 to 42 weeks, with a mean of 39.6 ± 1.5 weeks. Forty out of 59 processed units (67.8%) were derived from vaginal deliveries, and 19 were from cesarean sections. However, the presence of MSC-like cell clones correlated neither with the gestational age ($p = .50$) nor with the kind of delivery ($p = .38$).

However, both the time from collection to isolation and the volume of the UCB units were crucial for MSC-like cell generation (Table 2). Units stored for more than 15 hours failed to establish any MSC-like cells ($n = 15$). The mean

Table 1. Effect of FCS coating on the percentage of monocytes in the nonadherent fraction after plating and on the amount of adherent osteoclast-like cells and mesenchymal stem cell (MSC)–like cells by plating one half of the mononuclear cells in coated and the other half in uncoated plates

	Fetal calf serum–coated plates	Uncoated plates	*p* value
Monocytes (%)[a]	81.9 ± 10.6	59.7 ± 18.9	.01
Adherent cells[b]			
Wk 1	9.8 ± 9.7 (2–23)	8.5 ± 8.4 (1–20)	.86
Wk 2	1.7 ± 1.4 (0–4)	9.0 ± 7.4 (0–22)	.03
Wk 3	0.3 ± 0.5 (0–1)	13.8 ± 8.9 (3–24)	.05
Osteoclast-like cells[c] (U/U)	1/6	3/6	.12
MSC-like cells (U/U)	3/6 (4 colonies)	2/6 (2 colonies)	.56

$n = 6$; mean storage time of umbilical cord blood units, 10.7 ± 3.3 hours; range, 6 to 18 hours. p values were calculated using paired Student's *t*-test and c2 analysis.
[a]Mean percentage of monocytes recovered in the supernatant containing the nonadherent cells after overnight adherence.
[b]Mean value of cells, counted on three photomicrographs at a time. Photographs were taken at the respective time intervals. Range is indicated in parentheses.
[c]Osteoclast-like cells appeared in 70%–80% of the culture wells.

storage time of all processed UCB units was 10.2 ± 5.7 hours, ranging from 3 to 22 hours. Furthermore, a net volume of more than 33 ml placental blood and a MNC count greater than 1×10^8 were critical for isolation. Mean net volume was 42.8 ± 17.2 ml (range, 13 to 108 ml), and mean MNC count was $1.5 \pm 0.9 \times 10^8$ (range, 0.3 to 4.5). Three of the processed UCB units showed signs of coagulation, and six units showed signs of hemolysis. These units failed to generate MSC-like cells. By taking into account only those units having optimal quality, we were able to enhance the success rate from 29% to 63% (Table 2).

Discussion

The expected potential of mesenchymal progenitors is a matter of paramount importance for upcoming therapeutic strategies in the context of cellular therapy. However, although the biological characteristics of MSCs have been subject to intense investigation over the past few years, there is presently no universally accepted definition of this type of stem cell. Despite the lack of a definition, BM-derived MSCs have already been used in various preclinical and clinical studies [1–4]. However, the use of adult tissue-derived mesenchymal cells might not always be acceptable because of a significant drop in cell number and proliferative/differentiation capacity with age [5]. Therefore, UCB might result in an alternative for MSCs that is as good as has been proven for HSCs.

Although the evidence for the isolation of fibroblastoid cells with MSC characteristics from UCB is conflicting, our data reveal that MSC-like cells can be isolated from full-term UCB units with an efficiency of greater than 60%. Crucial parameters for achieving this yield were a storage time of less than 15 hours, a net volume of more than 33 ml, an MNC count greater than 1×10^8 MNCs, and no coagula or signs of hemolysis. It is tempting to speculate that using UCB units in line with blood-banking requirements will result in even higher yield in efficiency, which may intensify their use in therapeutic approaches.

The identification of MSC-like cells in UCB is in line with previously published results [11, 12]. On the other hand, other authors have denied the presence of MSC in UCB and claimed that only hematopoietic cells can be isolated from UCB [8–10]. Indeed, even in our experiments, a high percentage of UCB-derived cultures gave rise to monocyte-derived osteoclast-like cells. However, using FCS-coated culture plates enabled us to remove myeloid cells and osteoclast-like cells as their progeny. Besides, because it is known that FCS is crucial for the growth of MSCs [2], coated FCS might provide additional growth and adherence factors, favoring the generation of MSC-like cells from UCB.

The frequency of UCB-derived MSC-like cells was extremely low compared with BM. On the other hand, the clonal-derived MSC-like cells were able to generate much more progeny than the BM-derived cultures. MSC-like cells were fibroblastoid and expressed a typical set of MSC marker proteins, namely CD29, CD44, CD73, CD105, CD106, and HLA-class I, while lacking hematopoietic markers similar to BM-derived MSC. However, the expression of CD90, CD105, and CD106 differed between the

Table 2. Success rate in isolating mesenchymal stem cell (MSC)–like cells after recalculating umbilical cord blood (UCB) units fulfilling special criteria

Critical parameters	Success rate		p value
Total UCB units	17/59 (29 clones)	28.8%	
UCB units ≤15 h	17/44 (29 clones)	38.9%	.03
UCB units ≥33 ml	17/47 (29 clones)	36.2%	.03
UCB units $\geq 10^8$ MNC	16/40 (27 clones)	40.0%	.01
UCB units ≤15 h, ≥33 ml, $\geq 10^8$	17/38 (29 clones)	44.7%	.01
UCB units of optimal quality[a]	17/27 (29 clones)	63.0%	.01

Data represent the ratio of units that gave rise to MSC-like cells to the total number of units exploiting special characteristics. *p* values were calculated by χ^2 analysis comparing the frequency distributions of units according to critical quality parameters.
[a]≤15 hours, ≥33 ml, and $\geq 10^8$ MNC, fetal calf serum coat, no coagula, and no hemolysis, respectively.

two. Because at least CD106 expressed on MSC is functionally associated with hematopoiesis [21], the expression intensities may be related to the tissue the cells are derived from.

Just like BM MSCs, the MSC-like cells were also capable of differentiating towards the osteogenic and chondrogenic lineages. However, in contrast to MSCs, the MSC-like cells needed specific culture conditions to differentiate into the adipogenic lineage. The clonal cell isolation and the greater number of cell doublings may have caused an altered responsiveness in the MSC-like cells towards adipogenic induction, as indicated by Campagnoli et al. [22] and Goodwin et al. [12]. Furthermore, various publications have shown that BM-derived MSC populations are heterogeneous and that in multipotential clones the adipogenic potential is the first to be repressed [23]. On the other hand, the differing sensitivity to undergo adipogenic differentiation may also represent an inherent difference between the two cell populations. Because adipocytes, which are characteristic for adult human BM, are absent in fetal BM, the potential of MSCs to become adipocytes may be gained with aging to either occupy excess space or support hematopoiesis [24]. Therefore, the reduced sensitivity of MSC-like cells compared with BM-derived cells may be related to the ontogenetic age of the donors.

An open question is whether the MSC-like cells circulate or originate from the placenta. MSCs were isolated from first-trimester fetal blood and liver [22] and second-trimester fetal lung samples [25]. In addition, UCB of preterm deliveries seems to contain MSCs in relevant concentrations [11, 12]. However, we isolated MSC-like cells from more than 60% of the full-term UCB units. Because of the preparative regime of the blood harvest, those might be derived from the subendothelial layer of the cord, which has been shown to be an alternative source for MSCs [26]. However, if the presence of MSC-like cells was the result of lesions of the cord, contaminating endothelial cells within the cultures also should have been observed in high frequency in our experiments. But this was not the case.

To sum up, UCB can be used not only as an alternative source for hematopoietic cells but also for MSCs. MSC-like cells herein are characterized by their ability to proliferate in culture with an attached spindle-shaped morphology, by the presence of a consistent set of surface markers, and by their differentiation into at least three mesenchymal lineages under controlled in vitro conditions.

The capacity of MSCs to support hematopoiesis [1] and their potential to regulate immune responses [27] would commend their use in cotransplants to enhance the engraftment rate and quality of hematopoietic stem cells. Because hematopoietic stem cells as well as MSCs can be isolated from the same UCB, autologous cotransplantation may prove to be optimal for UCB transplantation approaches.

Acknowledgments

We gratefully acknowledge the collaborators in the obstetric departments in Mannheim, especially Dudi Vuthaj, Dr. J. Stöve from the Orthopedic Department of the University Hospital Mannheim for supplying BM harvests, and Daniela Griffiths for editing the manuscript. This work was supported by Forschungsfonds der Fakultät für Klinische Medizin Mannheim. Karen Bieback and Susanne Kern contributed equally to the results.

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