ISOLATION, CULTURE AND CHARACTERIZATION OF POSTNATAL HUMAN UMBILICAL VEIN-DERIVED MESENCHYMAL STEM CELLS

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ABSTRACT

On the basis of reports that mesenchymal stem cells (MSCs) can be isolated from the placenta/umbilical cord stroma, the present study was undertaken to isolate and characterize MSCs from the human umbilical cord veins. In this investigation, a cell population was isolated which was derived from the endothelium/subendothelium layers of 20 umbilical cord veins obtained from term deliveries using a solution of 0.1% collagenase type IV. Results suggest that these cells possess morphological, immunophenotypical and cell differentiation capacities similar to the bone marrow-derived mesenchymal stem cells (MSCs). The isolated cell population has fibroblastoid morphology which upon proper stimulation gives rise to adipocytes, osteocytes and chondrocytes in culture. Immunophenotypically, this cell population is positive for CD54, CD29, CD73, CD49e, CD166, CD105, CD13, and CD44 markers and alpha-smooth muscle actin and negative for CD31, CD45, CD49d, and CD34 markers, von Willebrand factor (vWF) and smooth muscle myosin (MySM). Altogether, these findings indicate that umbilical cord obtained from term deliveries is an important source of MSCs which could have an important application in cell therapy protocols.

Keywords: Mesenchymal stem cells, Umbilical cord, Cell differentiation

INTRODUCTION

Bone marrow is the main source of mesenchymal stem cells (MSCs) (1-3). These cells are a population of adhering cells derived from mononuclear BM cells having the capacity for self-renewal, supporting hematopoiesis, and differentiating into different cell lineages, such as adipocytes, osteocytes, chondrocytes, myocytes, cardiomyocytes, astrocytes and tenocytes (4-6). On the basis of these characteristics, many investigators have focused on the application of MSCs in cell based therapy protocols (7, 8). The number of bone marrow MSCs decrease significantly with age (9). Furthermore, there is a high risk of viral contamination during isolation of MSCs from bone marrow (10). Both these factors limit the use of bone marrow as a potential source of MSCs. In this regard, most attention should be focused on tissues containing cells with higher proliferative potency and differentiation capacity and lower risk for viral contamination. Umbilical cord blood (UCB) is known to be a rich source of hematopoietic stem cells (HSCs) that has practical advantages (11, 12), but the presence of MSCs in UCB has not yet been clearly resolved. It has been reported (13, 14) that UCB and mobilized adult blood do not contain MSCs. On the other hand, some reports (15, 16) have suggested that MSCs are present in several fetal organs and circulate in the blood of preterm fetuses simultaneously with hematopoietic precursors. What seems to be validated is that, UCB is enriched in pluripotent MSCs in the middle of gestation and these cells home after they leave circulation and the rest of them possibly deposit in placenta/umbilical cord stroma, including blood vessels (10). Recently, isolation of MSCs from the endothelium and subendothelium layers of the human umbilical cord have been described and their abilities for adipogenic and osteogenic differentiation have been demonstrated (10, 17). The objective of this investigation was to determine the presence of MSCs in the vascular endothelium of human umbilical cord vein of infants born at term, and to characterize them by flow cytometry, immuno-cytochemical and differentiation techniques.

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MATERIALS AND METHODS

Media antibodies and reagents

Dulbecco's modified Eagle's medium (DMEM), α-MEM and M199, Earle balanced salt solution (EBSS), antibiotics, collagenase IV, insulin, isobutyl-methylxantine, dexamethasone, indomethacin, β-glycerophosphate, ascorbate-phosphate, BMP-6, monoclonal antibodies for immunocytochemistry, fetal calf serum and other reagents were purchased from Sigma,USA. Monoclonal antibodies for immunofluorescent staining were purchased from Dako, Denmark, and were used at dilutions suggested by the supplier.

Samples, isolation and culture of MSCs

Umbilical cords (n = 20, gestational ages: 39-40 weeks, lengths: 15-20 cm) were obtained after normal or caesarian term deliveries from healthy infants under aseptic conditions and were carried to the lab in the filtered phosphate buffered saline (PBS) containing penicillin (300 U/ml), streptomycin (300µg/ml), gentamycin (150µg/ml) and fungizone (1µg/ml) and processed within 6-12 h. The umbilical veins were canulated and washed twice with EBSS containing 100 U/ml heparin. The distal ends were clamped and veins were filled with 0.1% collagenase type IV in M199 supplemented with antibiotics. After clamping the proximal ends, umbilical cords were incubated at 37º C for 20 min. The veins were washed with EBSS, followed by gentle massaging of the cords. The suspension of endothelial/ subendothelial cells were collected and centrifuged at 600 g for 10 min. The pellet was resuspended in DMEM supplemented with 100 U/ml penicillin, 100µg/ml streptomycin and 15 % FBS, after which the cells were plated onto 25 cm² tissue culture flasks (Nunc, Denmark) at a concentration of 10^3 cells/cm². Cultures were maintained at 37ºC in a humidified atmosphere containing 5% CO_2. Non-adherent cells were removed after 3 days by changing the medium, and adherent cells were kept in culture while were fed with fresh medium every 3 days, until the outgrowth of fibroblastoid cells which was 2 weeks later (10, 17). At that time, cultures were harvested with 0.05% trypsin- 0.02% EDTA and passaged (without dilution) into a new flask for further expansion or onto chamber slides (Nunc) for histochemical staining.

Flow cytometry studies

Expanded MSCs were detached from the culture flask by the use of PBS (pH = 7.4) containing trypsin (0.05%) and EDTA (0.02%), washed once with DMEM and once with filterated PBS. Cells were next suspended at a concentration of 1×10^6 cells in 50 µl of PBS and incubated for 45 min at 4°C in the dark with fluoresceine isothiocyanate (FITC) or phycoerythrin (PE) and following conjugated antibodies: anti-CD13-PE, anti-CD44-FITC, anti-CD45-FITC, anti-CD29-PE, anti-CD49d-PE, anti-CD49e-PE, anti-CD54-PE, anti-CD73-FITC, anti-CD166-FITC, anti-CD105-PE, anti-CD34-FITC, anti-CD31-FITC, anti-ASMA-FITC, anti-vWF-FITC and anti-MySM-FITC. In parallel, cells were incubated with an irrelevant Ab (anti-Aspergillus niger glucose oxidase, Dako) as a negative isotype control to exclude non-specifically labelled cells from the calculation. Upon completion of the incubation time, cells were washed twice with PBS supplemented with 2% bovine serum albumin (BSA)(Merck, Germany) and fixed with 1% paraformaldehyde (Sigma) solution in PBS (17). Analysis was next performed using a flow cytometer (FACsort, BD, USA). Befor each test, the percentage of viability that was more than 95% was measured with trypan-blue sataining and dead cells were counted with neobar slide.

Immunocytochemical studies

Part of primary cultures in flasks, as well as cells which were cultured on chamber slides washed with PBS, and fixed for 15 minutes at room temperature with 4% paraformaldehyde in PBS containing 0.1% Triton X-100 (Sigma, USA) or 1% paraformaldehyde in PBS for visualization of intracellular and surface antigens, respectively. After several washing with PBS and PBS-1% BSA, cells were incubated for 1 hour with the following monoclonal antibodies: anti –α smooth muscle actin (clone 1A4; Sigma; 1:400), anti-myosin (smooth)(clone HSM-V; Sigma; 1:500), anti-human PECAM-1 (CD31) (clone WM-59; Sigma; 1:100) and anti-human von willebrand factor (IgG fraction; Sigma; 1:800). Slides were next incubated with HRP (horse radish proxidase) conjugated anti mouse IgG secondary antibody (Amersham 1:300) and developed with DAB (Diamino benzidine) chromogen.

Differentiation methods

Assays of adipogenic and osteogenic differentiation were performed on the second passage cultures by plating 10^4 cells onto 3.6 cm² plates. For adipogenic differentiation, cells were grown to 100% confluence in growth medium supplemented with 10% FBS. Two-days post-confluent cells were incubated in adipogenesis-inducing medium (AIM) (DMEM, 4.5 g/L glucose, 1 µM dexamethasone, 200 µM indomethacin, 1.7 µM insulin, 500 µM isobutyl-methylxanthine, 10% FBS, 0.05 U/mL penicillin, and 0.05 µg/mL streptomycin) (Sigma) for 3 days, incubated 1 day in adipogenesis maintenance medium (AMM) (DMEM, 4.5 g/L glucose, 1.7

µM insulin, 10% FBS, 0.05 U/mL penicillin, and 0.05 µg/mL streptomycin) and then switched to AIM again. After the third cycle, cells were fed with AMM for up to 21 days of differentiation (18). Osteogenic differentiation was induced in 2 weeks by α-MEM supplemented with 0.1 µM dexamethasone, 10 µM β-glycerophosphate and 50 µM ascorbate-phosphate (all from Sigma) (17, 18). Differentiated cells were identified by histochemical staining. For adipocytes identification, intracellular lipid accumulation was visualized using Oil Red-O-staining. Briefly, cells were fixed in 10% solution of formaldehyde in aqueous phosphate buffer for more than one hours, washed with 60% isopropanol, and stained with Oil Red- O-solution (in 60% isopropanol) for 10 min, followed by repeated washing with water before being destained in 100% isopropanol for 15 min. The optical density (OD) of the solution was measured at 500 nm (19). Osteocytes were identified by the alkaline phosphatase (ALP) activity (alkaline phosphatase staining kit, sigma cat. 86R). Control cultures without the differentiation stimuli were maintained in parallel to the differentiation experiments and stained in the same manner. For chondrogenic stimulation, MSCs were plated at 50 cells/cm^2 and cultured in complete media for 7 days. In this way, a micromass culture system was used (20). Approximately 200,000 MSCs were placed in a 15-ml polypropylene tube (Falcon; Bendford, MA) and pelleted into micromasses by centrifugation at 450 g for 10 minutes.The pellet was cultured for 21 days in chondrogenic media that contained 500 ng/ml BMP-6 (sigma) rich in glucose (25 mM) and DMEM supplemented with 10 ng/ml transforming growth factor beta, 3×10^{-7} M dexamethasone, 50µg/ml ascorbate-phosphate, 40µg/ml proline, 100µg/ml pyruvate and 50µg/ml ITS^{+tm} Premix (Becton Dickinson). For microscopic examinations, the pellets were embedded in paraffin, cut into 5 µm sections, and stained with 1 % toluidine blue (sigma) and 1% sodium borate (sigma) for 5 minutes (21).

RESULTS

Immunocytochemical staining on isolated cells

Initially, two types of adherent cells were observed in primary cultures: Endothelial cells (ECs) with small flat morphology and a few spindle-shape fibroblastoid cells which were identified primarily as MSCs and observed between ECs (figure 1 a).

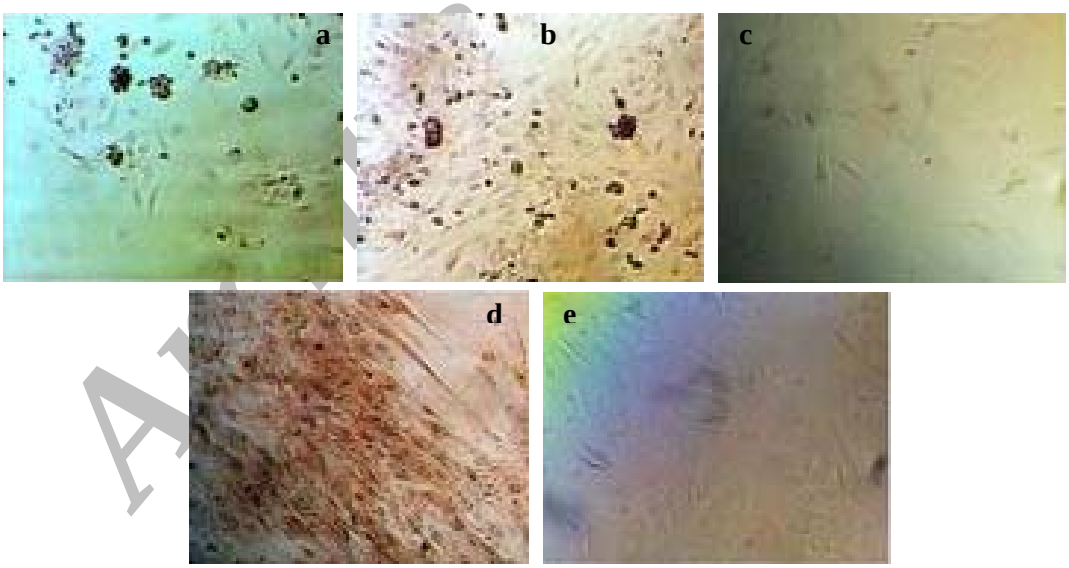

Figure 1. Morphology and immunocytochemical characteristics of cells isolated from the human umbilical vein endothelial/subendothelial layer.

Primary cultures containing MSCs and endothelial cells or Ecs are stained in (a) and (b) with anti-vWf and anti-PECAM1 (CD31) antibodies respectively. Obviously, clear DAB signal for ECs (compact round cells) indicate that they are vWF and PECAM1 (CD31)-positive, while MSCs (spindle shape cells) remined pinkish and so they are vWF and PECAM1 negative.

Immunocytochemical staining with anit-MySM antibody (c) and anti-ASMA antibody (d) show that isolated MSCs are MySM negative, while they were ASMA-positive (e). In the second cell passage (e), the MSC cultures appeared to be homogeneous and predominant cell type.

Final magnifications for a and b were ×100 and for c,d and e were ×200.

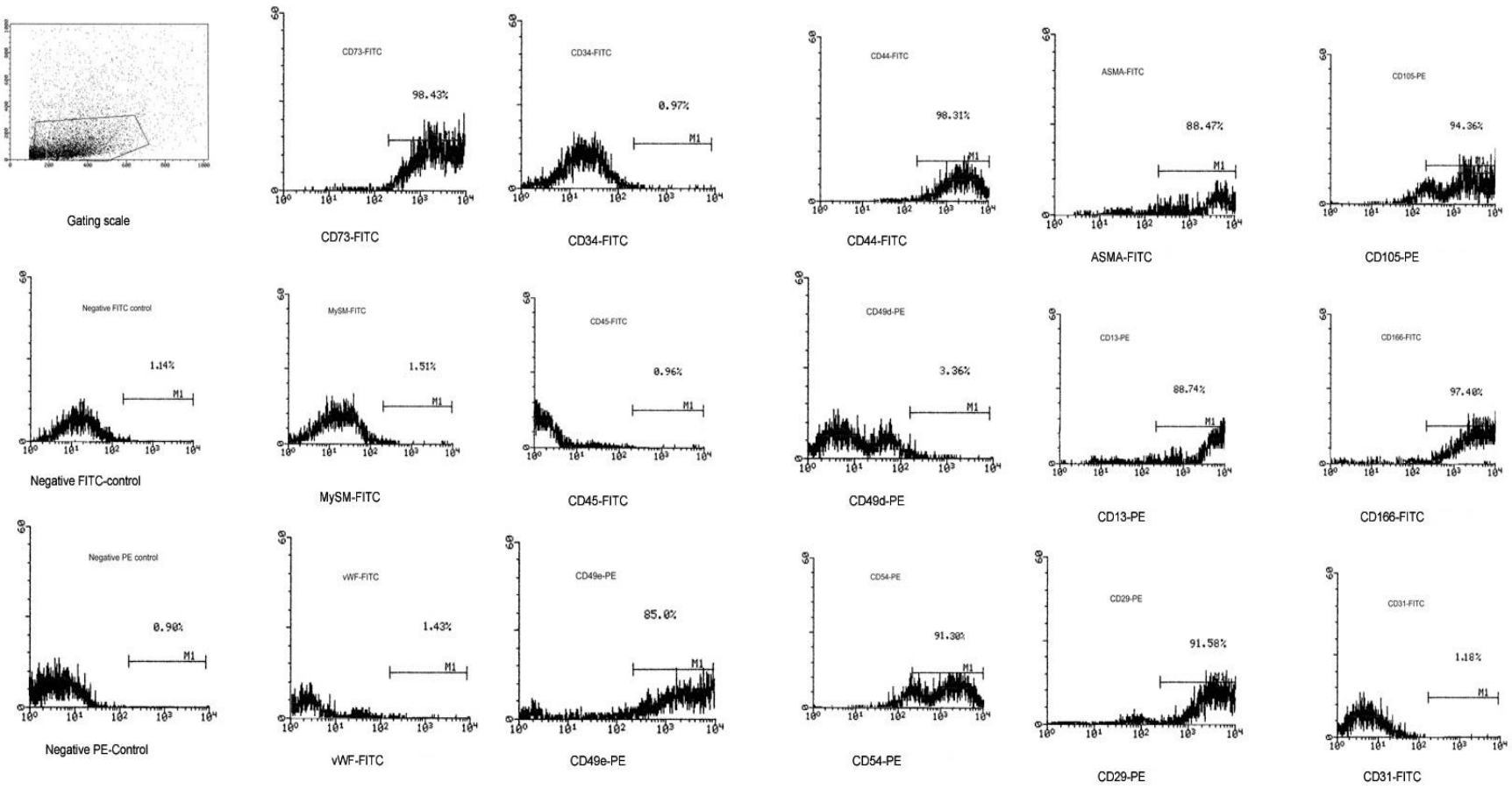

Figure 2. Flow cytometry histograms show the immunophenotype of MSCs isolated from the postnatal human umbilical vein. MSCs were positive for CD29, CD13, CD44, CD49e, CD54, CD166, CD73, CD105 and ASMA. These cells were negative for CD49d, CD31, CD45, CD34, vWF and MySM.

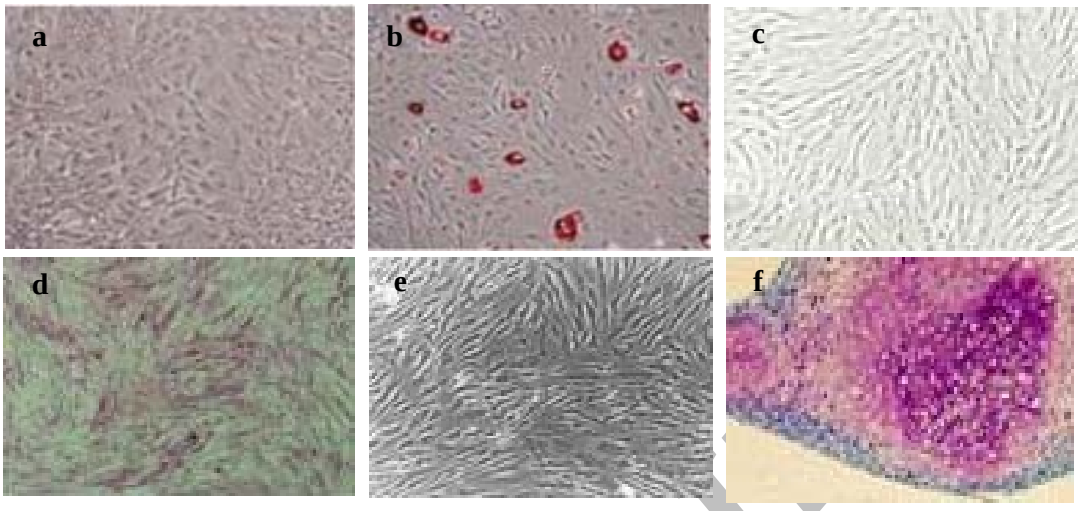

Figure 3. Differentiation potential of postnatal human umbilical vein-derived mesenchymal stem cells. (a) and (b) show the results of Oil Red O staining in control untreated medium, and in cell cultures growing within 2 weeks in adipogenic medium, respectively. Results of osteogenic differentiation showing non-stimulated (c) and stimulated cells (d) stained with diagnostic alkaline phosphatase kit. (e) and (f) show the results of toluidine blue-sodium borate staining in control untreated MSCs and in MSC cultures exposured to chondrogenic medium for 21 days, respectively. Final magnifications for (a), (b), (c), (d) and (e) are ×100 and for (f) is ×40 .

Immunocytochemical staining showed that ECs were vWF and PECAM1 (CD31) positive, while MSCs were negative for both of them (figure 1 a,b). Furthermore, MSCs were MySM negative, while they were ASMA positive (figure 1 c,d). After culture for 2 weeks, these MSCs became the predominant cell type, while the endothelial cells remained compact and did not spread, migrate or proliferate. vWF and PECAM1 positive cells (endothelial cells) did not exceed 0.5-1% of the total cell number. In the second cell passage, the MSC cultures with a high replicative potential were more homogeneous (figure 1 e).

Flow cytometry results

In the cytomeric analyses, MSCs were positive for the following adhesion molecules: CD29 (integrin β1), CD13 (aminopeptidase), CD44 (H-CAM), CD49e (integrin α5), CD54 (ICAM-1), CD166 (ALCAM), and also were positive for CD73 (5-terminal nucleotidase), CD105 (Endoglin) and ASMA (α-smooth muscle actin) whereas they were negative for CD49d (integrin α4), CD31 (PECAM1), CD45 (leukocyte common antigen), CD34, vWF (von Willebrand factor) and MySM (smooth muscle myosin) (figure 2).

Differentiation results

Differentiation studies performed on MSCs revealed their potential to differentiate into adipocytes, osteoblasts and chondrocytes. MSC cultures in adipogenic differentiation medium, after 7 days, led to the appearance of larger round cells presenting numerous fat vacuoles in the cytoplasm. These lipid droplets were oil red-O-positive while untreated control cultures did not have lipid droplets in their cytoplasms. (figure 3 a,b). Similarly osteogenic stimulation of MSCs led to the appearance of refringent crystals in the cells after 2 weeks. By the end of the second week of stimulation, most of the MSCs also became alkaline phosphatase-positive. Untreated control cultures growing in regular medium without any osteogenic differentiation stimuli did not show spontaneous osteoblast formation even after 3 weeks of cultivation (figure 3 c,d). MSC cultures in chondrogenic medium after 21 days led to formation of cartilage structures that can be distinguished specifically by staining with toluidine blue-sodium borate. These structures did not form in untreated control cultures after this period.

DISCUSSION

The isolation of a cell population derived from the endothelium/subendothelium layers of the umbilical cord vein is described The immunophenotypical and morphological profiles of these cells are the same as those of MSCs isolated from bone marrow (22-24). Recently, isolation of cells from the endothelium and subendothelium layers of the umbilical cord morphologically similar to those which were isolated in this investigation (10, 17) and their abilities for adipogenic and osteogenic differentiation have been reported. However

present investigation is one of the first studies for isolation of cells with these characteristics from the endothelium/subendothelium layers of the human umbilical vein which were characterized extensively by immunophenotypical and immunocytochemical studies. Although the biochemical markers which were used for identification of cells from endothelium and subendothelium layers of umbilical cord (10, 17) were different from those that were employed in this study, the cell type is the same in view of the noted similarities, including cell adherence and fibroblastoid morphology. One of the reported methods for isolation of MSCs from the endothelium/subendothelium layers of the umbilical cord vein has used 1X endothelial cell growth factor (EGF) and vascular endothelial growth factor (VEGF) for enrichment of culture medium (17). However, cells which have been isolated by this method can not used widely in the clinical trials until effects of EGF and VEGF on the cells to be more defined (12). In this study, MSCs from the endothelium and subendothelium layers of umbilical cord vein were isolated and cultured without using these materials. Nevertheless, additional studies are required for further characterization of the pluripotency of these cells in accordance with the pluripotency of bone marrow-derived MSCs. Umbilical cord/placenta vessels may serve as a rich source of MSCs for experimental and clinical demands if the multilineage differentiation capability of these cells is documented.

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REFERENCES

1. Deans RJ, Moseley AB. Mesenchymal stem cells: biology and potential clinical use. Exp Hematol 2000; 28:875-884.
2. Minguell JJ, Conget P, Erices A. Biology and clinical utilization of mesenchymal progenitor cells. Braz J Med Biol Res 2000; 33:881-887.
3. Conget PA, Minguell JJ. Phenotypical and functional properties of human bone marrow mesenchymal progenitor cells. J Cell Physiol 1999; 181:67-73.
4. Minguell JJ, Erices A, Conget P. Mesenchymal stem cell. Exp Biol Med 2001; 226:507-520.
5. Quesenberry PJ, Abedi M, Aliota J, Colvin G, Demers D, Dooner M, Greer D, Hebert H, Menson MK, Pimentel J, Paggioli D. Stem cell plasticity: an overview. Blood Cells Mol Dis 2004; 32:1-4.
6. Lakshmipathy U, Verfaillie C. Stem cell plasticity. Blood Rev 2005; 19:29-38.
7. Barry FP, Murphy JM. Mesenchymal stem cells: Clinical applications and biological characterization. Int J Biochem Cell Biol 2004; 36:568-584.
8. Caplan AL, Bruder SP. Mesenchymal stem cells: building block for molecular medicine in the 21 st century. Trends Mol Med 2001; 7:259-264.
9. Rao MS, Mattson MP. Stem cells and aging: expanding the possibilities. Mech Aging Dev 2001; 122:713-734.
10. Romanov YA, Svintsitskya VA, Smirnov VN. Searching for alternative sources of postnatal human mesenchymal stem cells: Candidate MSC-like cells from umbilical cord. Stem Cells 2003; 21:105-110.
11. Punzel M, Ho AD. Divisional history and pluripotency of human hematopoietic stem cells. Ann N Y Acad Sci 2001; 938:72-82.
12. Braker JN, Wagner JE. Umbilical cord blood transplantation: Current practice and future innovations. Crit Rev Oncol Hematol 2003; 48:35-43.
13. Wexler SA, Donaldson C, Dening-Kendall P, Rice C, Bradley B, Hows JM. Adult bone marrow is a rich source of human mesenchymal stem cells but umbilical cord and mobilized adult blood are not. Br J Haematol 2003; 121:368-374.
14. Mareschi K, Biasin E, Piacibello W, Aglietta M, Madon E, Faioli F. Isolation of human mesenchymal stem cells: Bone marrow versus umbilical cord blood. Haematologica 2001; 86:1099-1100.
15. Campagnoli C, Roberts IA, Kumar S, Bennett PR, Bellantuono I, Fisk NM. Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver and bone marrow. Blood 2001; 98:2396-2402.
16. Erices A, Conget P, Minguell JJ. Mesenchymal progenitor cells in human umbilical cord blood. Br J Haematol 2000; 109:235-242.
17. Covas DT, Siufi JLC, Silva ARL, Orellana MD. Isolation and culture of umbilical vein mesenchymal stem cells. Braz J Med Biol Res 2003; 36:1179-1183.

18. Janderova L, McNeil M, Murrell AN, Mynatt RL, Smith SR. Human Mesenchymal stem cells as an in vitro model for human adipogenesis. Obesity Res 2002; 11:65-74.
19. Ramirez-Zacarias JL, Castro-Munozledo F, Kuri-Harcuch W. Quantitation of adipose conversion and triglycerides by staining intracytoplasmic lipids with Oil Red O. Histochem 1992; 97:493-497.
20. Johnstone B, Hering TM, Caplan AI et al. In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells.Exp Cell Res 1998;238:265-272.
21. Ichiro S, Benjamin LL, Jason R et al. Expansion of human adult stem cells from bone marrow stroma: Conditions that maximize the yields of early progenitors and evaluate their quality. Stem cells 2002;20:530-541.
22. Friedenstein AJ, Deriglazoa UF, Kulagina NN. Precursors for fibroblast in different populations of hematopoietic cells as detected by the in vitro colony assay method. Exp Hematol 1974; 2:83-92.
23. Friedenstein AJ, Gorskaja JF, Kulagina NN. Fibroblast precursors in normal and irradiated mouse hematopoietic organs. Trend Mol Med 1976; 4:267-274.
24. Majumdar MK, Thiede MA, Mosca JD, Moorman M, Gerson SL. Phenotypic and functional comparison of cultures of bone marrow-derived mesenchymal stem cells (MSC) and stromal cells. J Cell Physiol 1998; 176:57-66.