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Characterization of two populations of mesenchymal progenitor cells in umbilical cord blood

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Abstract

Umbilical cord blood (UCB) is a valuable source for hematopoietic progenitor cell therapy. Moreover, it contains another subset of non-hematopoietic population referred to as mesenchymal progenitor cells (MPCs), which can be ex vivo expanded and differentiated into osteoblasts, chondrocytes and adipocytes. In this study, we successfully isolated the clonogenic MPCs from UCB by limiting dilution method. These cells exhibited two different morphologic phenotypes, including flattened fibroblasts (majority) and spindle-shaped fibroblasts (minority). Both types of MPCs shared similar cell surface markers except CD90 and had similar osteogenic and chondrogenic potentials. However, the spindle-shaped clones possessed the positive CD90 expression and showed a greater tendency in adipogenesis, while the flattened clones were CD90 negative cells and showed a lower tendency in adipogenesis. The high number of flattened MPCs might be linked to the less sensitivity of UCB-derived MPCs in adipogenic differentiation.

Keywords: Mesenchymal progenitor cells; Clonogenic; Differentiation

1. Introduction

During development, hematopoiesis is migratory, occurring at several sites in the body of the developing fetus before confining itself to the bone marrow. This implies that both hematopoietic progenitor cells and their stromal supporting cells could exist in the circulatory system of prenatal fetus. Besides hematopoietic stem/progenitor cells, umbilical cord blood (UCB), similar to bone marrow, has been demonstrated to contain mesenchymal stem cells/mesenchymal progenitor cells (MPCs) (Erices et al., 2000; Lee MW, et al., 2004). MPCs were initially referred to as plastic-adherent cells in bone marrow that formed fibroblastic colonies in vitro (Friedenstein et al., 1974). Currently, MPCs are found in many different tissues and can be expanded ex vivo in large quantities and induced to differentiate into cells of mesodermal lineage, such as osteoblasts, chondrocytes and adipocytes (Barry and Murphy, 2004; Pittenger et al., 1999; Erices et al., 2000; Goodwin et al., 2001). Lee OK, et al. (2004) reported that UCB contained a more primitive population of multipotent MPCs, which could differentiate into cells of three germ layers. However, two different phenotypic clones of MPCs are found in bone marrow and placenta, which are flattened fibroblasts and spindle-shaped fibroblasts, and these clonogenic MPCs have similar surface marker expression (Muraglia et al., 2000; Fukuchi et al., 2004). It is not clear that if these two types of clonogenic MPCs possess the same mesenchyme-lineage differentiation capability. We are trying to explore whether these two types of clonogenic MPCs exist in UCB and assess their differentiation potentials in mesenchymal lineages. In this study, we isolated two different types of MPCs

Abbreviations: UCB, umbilical cord blood; MPCs, mesenchymal progenitor cells.

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from UCB at clonal level, and their surface marker profiles and differentiation potentials were comparatively analyzed further.

2. Materials and methods

2.1. Clonogenic MPCs isolation and flow cytometric analysis

Term UCB was harvested with a standard 250-ml blood bag (Terumo, Shibuya-ku, Tokyo, Japan) with informed consent and processed within 24 h. MPCs were isolated by Ficoll-Paque density centrifugation (1.077 g/ml, Amersham, Uppsala, Sweden) and cultured in Minimum Essential Medium alpha-modification (α-MEM, Hyclone, Logan, UT) containing 20% fetal bovine serum (FBS, Hyclone), 4 ng/ml β-FGF (R&D Systems, Minneapolis, MN), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma, St. Louis, MO) according to the method described previously (Erices et al., 2000). To obtain single cell-derived MPCs, the first passage MPCs were cultured onto 96-well plate (Corning, Acton, MA) by limiting dilution (Lee OK, et al., 2004). The clonogenicity of the first passage MPCs samples was about 15%. The clonogenic MPCs were expanded at a split ratio 1:4 as follows. For surface markers analysis, cells at passage 6 were trypsinized and suspended in phosphate buffer saline (PBS, Gibco BRL). Primary antibodies against human antigens: CD26, CD29, CD31, CD34, CD44, CD45, CD90 (Thy-1), HLA-A, B, C, and HLA-DR were purchased from Becton–Dickinson (San Jose, CA), and SH2, SH3 and SH4 were purified from respective hybridoma cells acquired from American Type Culture Collection (Manassas, VA). The non-specific mouse IgG (Becton–Dickinson) was substituted for the primary antibodies as isotype control and anti-mouse IgG-FITC (Beckman Coulter, Brea, CA) was used as the secondary antibody for staining. Data were analyzed using a FACScan flow cytometry system (Becton–Dickinson).

2.2. In vitro differentiation

Clonogenic MPCs cells were cultured to confluence for osteogenic and adipogenic differentiations and over-confluence for chondrogenic differentiation for 3 weeks. The in vitro differentiations were performed by α-MEM supplemented with 10% FBS, 0.1 μM dexamethasone (Sigma), 10 mM β-glycerolphosphate (Sigma), 50 μM ascorbic acid (Sigma) for osteogenesis, α-MEM supplemented with 10% FBS, 1 μM dexamethasone (Sigma), 0.5 mM methyl-isobutylxanthine (Sigma), 10 μg/ml insulin (Invitrogen, Carlsbad, CA), 100 μM indomethacin (Sigma) for adipogenesis and α-MEM supplemented with 10 ng/ml TGF-β1 (PeproTech, Rocky Hill, NJ) for chondrogenesis. Osteogenic potential was assessed by von Kossa staining method, chondrogenic potential was evaluated by the staining of proteoglycan with Safranin O (Sigma), and adipogenic potential was observed by staining with Oil Red O (Sigma). For quantification of adipogenic differentiation, ethanol was added to each well to extract the Oil Red O from the cells. The amount of Oil Red O released was determined spectrophotometrically at 550 nm with a reference of 650 nm and compared to an Oil Red O standard titration curve (in 't Anker et al., 2003). For detecting the mRNA expression, total RNA was isolated using Trizol reagent (MRC, Cincinnati, OH), and the complementary DNA (cDNA) was synthesized by ImPro-II reverse transcriptase (Promega, Madison, WI) with oilgo-dT primer. The primer sequences used were as follows: β-actin forward: 5′-TGTGGATCAGC AAGCAGGAGTA-3′, reverse: 5′-CAAGAAAGGGTGTAACGCAACTAAG-3′; PPARγ2 forward: 5′-CCAGAAAATGACAGACCTCAGACA-3′, reverse: 5′-GCAGGAGCGGGTGAAGACT-3′. The relative expression level of β-actin was used as an internal control to normalize PPARγ2 gene expression in each sample. Real-time PCR was performed by ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) with SYBR Green PCR master mix (Applied Biosystems).

3. Results and discussions

Clonogenic MPCs with different phenotypes were observed in human bone marrow and placenta (Muraglia et al., 2000; Fukuchi et al., 2004). In this study, we successfully established 56 clones with high proliferation capability from 10 UCB units. Among them, two different morphologic phenotypes were observed: flattened fibroblastic clones (93%) and spindle-shaped fibroblastic clones (7%) (Fig. 1A, B). The growth rates were similar between flattened MPCs (28.6 ± 3.4 h) and spindle-shaped MPCs (30.4 ± 2.5 h) calculated during passages 4–6. Both types of clonogenic MPCs showed a high proliferative capacity, which were passed over 10 passages. Interestingly, the ratio of these two different phenotypic MPCs in UCB was significantly different from that in bone marrow (no data for placenta). At the clonogenic level, MPCs with spindle-shaped phenotype are highly abundant in bone marrow, while flattened MPCs are rare (Muraglia et al., 2000). The physiological interpretation of the difference between these two types of MPCs is unclear, but it implies that the differences of microenvironment might be an important factor between UCB and bone marrow.

The cell surface markers of these two types of MPCs were examined by FACS analysis. As shown in Fig. 2, both types of MPCs were negative for CD34, CD26, CD31, CD45 and HLA-DR. Both were positive for mesenchymal progenitor cell markers SH2, SH3 and SH4, adherent molecules CD29, CD44 and HLA-A, B, C. These surface marker profiles are consistent with previously reported UCB- and bone marrow-derived MPCs (Goodwin et al., 2001; Pittenger et al., 1999). However, CD90 was differently expressed by these two cell populations. Spindle-shaped clonogenic MPCs expressed a high level of CD90, while flattened clonogenic MPCs showed negative expression of CD90. These data might explain the inconsistent results in CD90 expression of UCB-derived MPCs in different reports (Erices et al., 2000; Goodwin et al., 2001; Bieback et al., 2004). It suggests that different levels of CD90 expression in UCB-derived MPCs may be related to the percentage of these two populations in heterogeneous culture condition. This result was consistent with the findings in murine lung fibroblasts in which two populations were identified, one was spindle-shaped and CD90 positive fibroblasts, and the other was rounded and CD90 negative fibroblasts (Phipps et al., 1989; Penney et al., 1992). Furthermore, CD90 has been known as a negative regulator for hematopoietic proliferation (Mayani and Lansdrop, 1994). It was also reported that hematopoietic progenitor cells from UCB possessed higher proliferation and expansion potential than that from bone marrow (Mayani and Lansdrop, 1998). The lower frequency of $CD90^+$ MPCs might provide a more beneficial environment for the proliferation of hematopoietic progenitor cells in cord blood.

The differentiation potentials of different types of clonogenic MPCs were investigated further. Results showed that both types of clonogenic MPCs could differentiate into osteogenic and chondrogenic lineages under appropriate conditions (Fig. 1C–F). However, in adipogenic induction, the spindle-shaped MPCs exhibited many typical neutral lipid vacuoles within the cells as mature adipocytes (Fig. 1H), while the flattened MPCs only contained sparsely small lipid droplets or even no lipid droplets at all (Fig. 1G). We further quantified

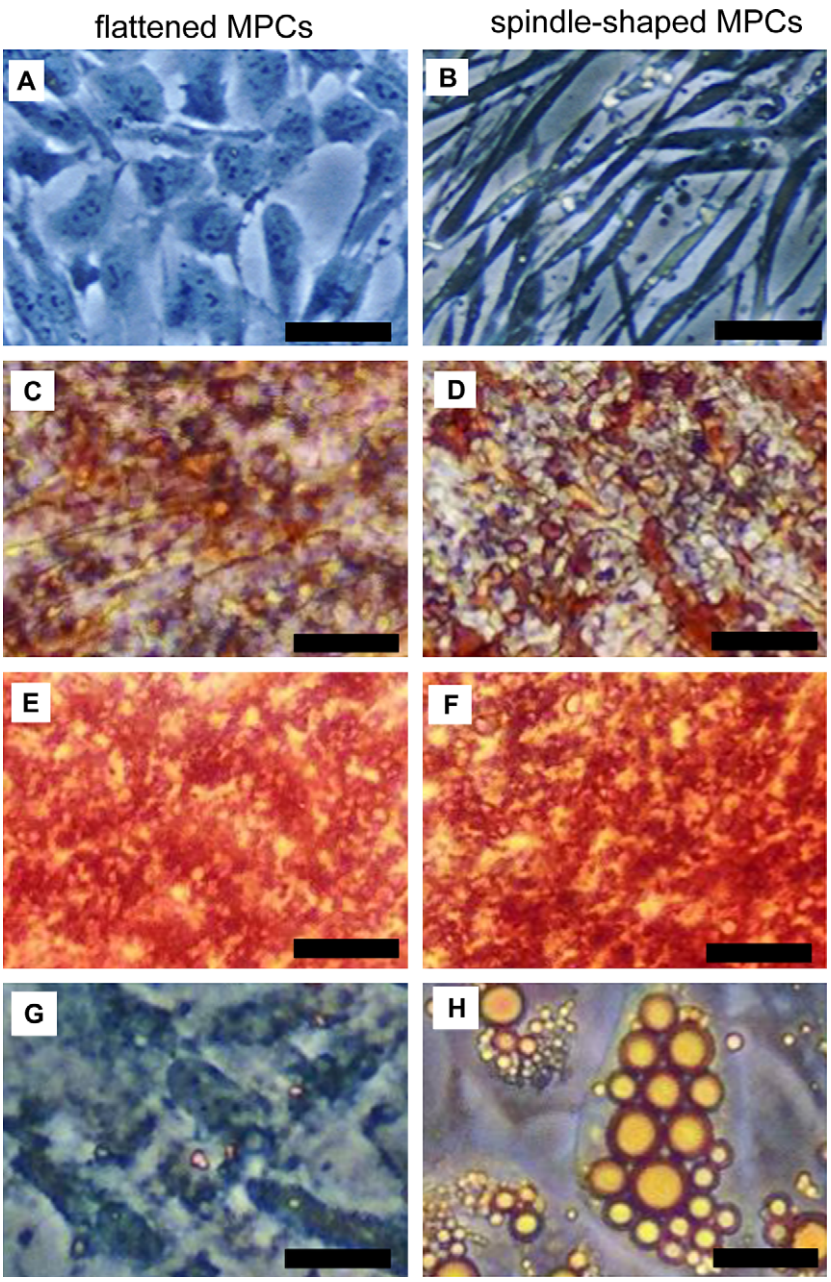

Fig. 1. Morphology and differentiation potentials of two types of clonogenic MPCs from umbilical cord blood. Flattened fibroblastic phenotype (A) and spindle-shaped fibroblastic phenotype (B). Both types of MPCs were exposed in vitro to differentiation medium for 3 weeks. The osteogenic differentiation was assessed by von Kossa staining showing the presence of matrix mineralization (C, D), the chondrogenic differentiation was stained positively in proteoglycan using Safranin O (E, F), and adipogenic differentiation was assayed by Oil Red O staining at lipid vacuoles (G, H). The flattened clonogenic MPCs showed a low tendency in adipogenic differentiation. Bar scales: 50 μm.

the intracellular triacylglycerol accumulation between both types of clonogenic MPCs. As shown in Fig. 3A, the amount of cell-bound Oil Red O in spindle-shaped MPCs was 5.3-fold higher than that found in flattened MPCs during adipogenesis. The adipogenic transcription factor, PPARγ2, in spindle-shaped MPCs was expressed higher than that expressed in flattened MPCs by 1.6-fold (Fig. 3B). It was reported that UCB-derived MPCs showed a reduced capability to undergo adipogenesis (Bieback et al., 2004). Recently, we have also found that UCB-derived MPCs have lower adipogenic potential than bone morrow-derived MPCs in vitro (Chang et al., 2006). It was demonstrated that CD90 could serve as a marker of preadipocytes in 3T3-L1 cells, and the $CD90^{+}$ subpopulation was lipid-containing cells within lung fibroblasts (Gagnon et al., 2004; Phipps et al., 1989). Our data suggested that high number of flattened MPCs might actually be linked to the less sensitivity of UCB-derived MPCs in adipogenic differentiation. Although the nature of adipogenesis from MPCs was unknown in vivo, the ratio between flattened MPCs and spindle-shaped MPCs in different tissues, including UCB and adult bone marrow, may account for their physiology in terms of adipogenic development.

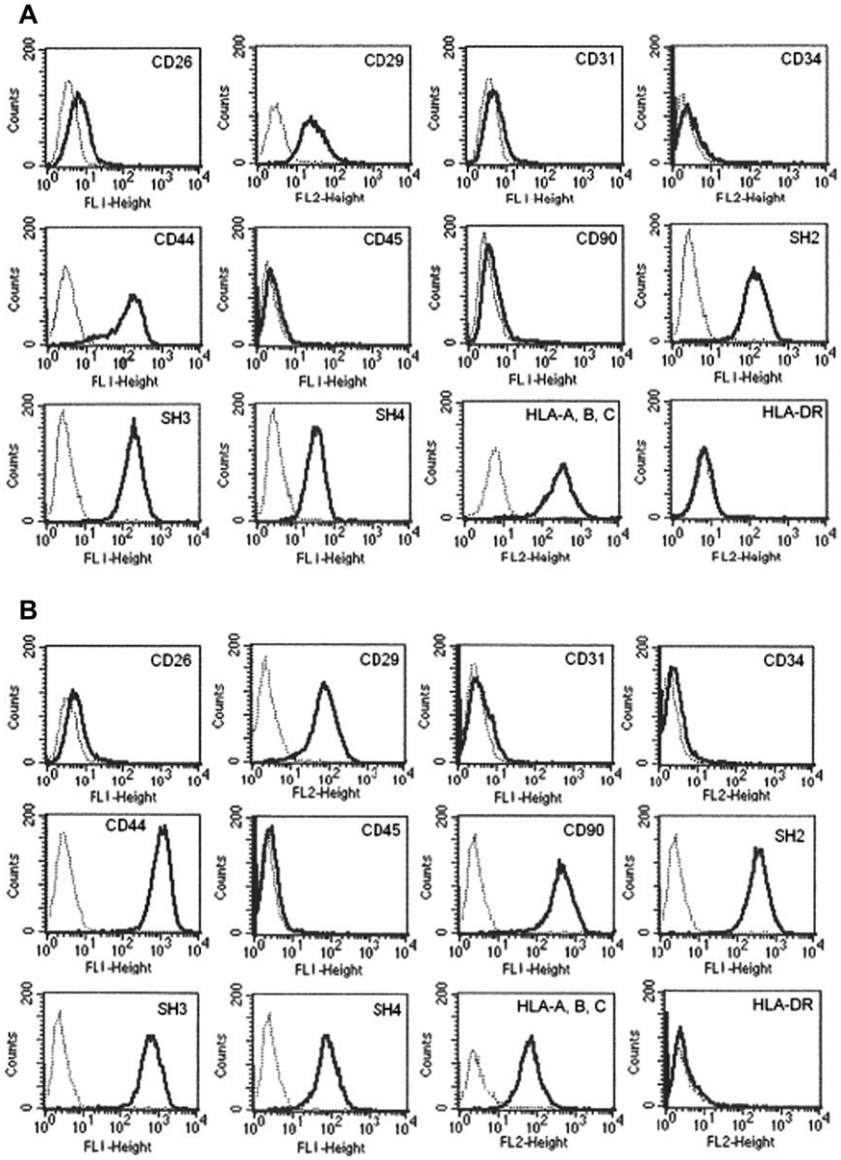

Fig. 2. Comparison of cell surface marker profiles between two types of clonogenic MPCs. Flattened fibroblastic MPCs (A) and spindle-shaped fibroblastic MPCs (B). Both types of MPCs at passage 6 were analyzed by flow cytometry with antibodies against the indicated antigens. The respective isotype control was shown in dotted line. The flattened clonogenic MPCs showed negative expression of CD90, while the spindle-shaped clonogenic MPCs expressed a high level of CD90.

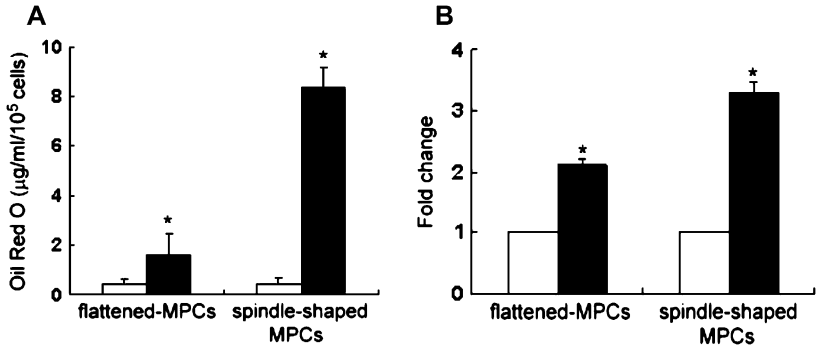

Fig. 3. Adipogenic capacity of two types of clonogenic MPCs. The adipogenic capacity was represented by the extraction of cell-bound Oil Red O, which was normalized by the cell number in a panel of wells in parallel (A). The PPARγ2 gene expression in both type of clonogenic MPCs was detected by real-time PCR at the third week of induction (B). The data were represented as fold changed in differentiated cells relative to the corresponding undifferentiated cells. Undifferentiated: white bar, Adipogenic induction at the third week: black bar . Results represented mean ± SD of three replicas and derived from at least two independent experiments. Asterisks indicate statistically significant difference ($p < 0.05$) compared to undifferentiated condition.

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