Isolation of multipotent mesenchymal stem cells from umbilical cord blood

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It is well accepted that umbilical cord blood (UCB) is a source for hematopoietic stem cells, and transplantation of cord blood has been part of clinical practice for more than 10 years. However, controversy exists as to whether UCB contains mesenchymal stem cells (MSCs), which are capable of differentiating into cells of different connective tissue lineages such as bone, cartilage, and adipose tissue and are the best candidates for tissue engineering of different connective tissue lineages such as bone, cartilage, and fat, and little success has been reported in the literature about the isolation of such cells from cord blood. Here we report a novel method to obtain single cell-derived, clonally expanded mesenchymal stem cells that are of multilineage differentiation potential by negative immunoselection and limiting dilution. The immuno-phenotype of these clonally expanded cells is consistent with that reported for bone marrow mesenchymal stem cells. Under appropriate induction conditions, these cells can differentiate into bone, cartilage, and fat. Surprisingly, these cells were also able to differentiate into neuroglial- and hepatocyte-like cells under appropriate induction conditions and, thus, these cells may be more than mesenchymal stem cells as evidenced by their ability to differentiate into cell types of all 3 germ layers. In conclusion, umbilical cord blood does contain mesenchymal stem cells and should not be regarded as medical waste. It can serve as an alternative source of mesenchymal stem cells to bone marrow. (Blood. 2004;103:1669-1675)

Introduction

It is well accepted that umbilical cord blood (UCB) is a source for hematopoietic stem cells, and transplantation of cord blood has been part of clinical practice for more than 10 years.1-3 However, controversy exists as to whether UCB contains mesenchymal stem cells (MSCs), which are capable of differentiating into cells of different connective tissue lineages such as bone, cartilage, and adipose tissue and are the best candidates for tissue engineering of musculoskeletal tissues. To date, the most common source of MSCs has been the bone marrow, but aspirating bone marrow from the patient is an invasive procedure and, in addition, it has been demonstrated that the number and the differentiating potential of bone marrow MSCs decreases with age. Therefore, the search for alternative sources of MSCs is of significant value. So far, little success has been reported in the literature about the isolation, characterization, and differentiation of MSCs from UCB. Erices et al reported that UCB-derived mononuclear cells gave rise to 2 adherent cell types, one of which expressed MSC-related surface antigens. Mareschi et al reported that in a given condition, it was possible to isolate MSCs from bone marrow but not UCB. Goodwin et al recently reported multilineage differentiation ability by cells isolated from UCB, which express bone, fat, and neural markers. In these 2 reports, neither Erices et al nor Goodwin et al provided sufficient evidence to fulfill the qualifying criteria for MSCs because relatively heterogeneous cells were reported by both groups.

Because the surface phenotype of MSCs has been clearly characterized and reported, which should be lineage-marker negative, it would be reasonable to deplete all these lineage-committed cells, such as CD3, CD7, CD19, CD38, and glycoporphin A+ cells to get MSCs. Even with such cell enrichment procedures, the resulting cell population is still heterogeneous and it is imperative to obtain clonally expanded MSCs to demonstrate their multipotentiality. Most important of all, the proliferation and differentiation abilities of MSCs may decrease with the donor age. The cells contained in UCB can be considered as “very young” and, thus, UCB may be an excellent alternative source of MSCs. Unfortunately, most of the time UCB is still regarded as medical waste in the delivery rooms. Therefore, the aim of this study is to investigate the possibility of obtaining clonally expanded MSCs that have the potential for multilineage differentiation. We hypothesized that MSCs, which are multipotent, exist in UCB and can be isolated and expanded in culture.

Materials and methods

Cytokines

Nerve growth factor (NGF), epidermal growth factor (EGF), vitronectin, and transforming growth factor β (TGF-β1) were from Becton Dickinson (Flanders, NJ). Acidic and basic fibroblast growth factor (aFGF, bFGF), sonic hedgehog (SHH), brain-derived growth factor (BDNF), hepatocyte growth factor (HGF), and oncostatin M (OSM) were from R&D Systems (Minneapolis, MN).

Antibodies

Antibodies against human osteopontin, human microtubule-associated protein 2 (MAP-2), human glial fibrillary acidic protein (GFAP), and human type II collagen were purchased from Santa Cruz Biotechnology (grant no. VGH 91-390-3). Reprints: Oscar Kuang-Sheng Lee, Department of Orthopaedics and Traumatology, Veterans General Hospital-Taipei, 201, Section 2, Shi-Pai Rd, Taipei 11221, Taiwan; e-mail: kslee@vghtpe.gov.tw.

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Osteogenic differentiation. In vitro differentiation of MSCs was performed by plating the cells on gelatin-coated dishes in alpha-MEM supplemented with 10% FBS, 100 ng/mL bFGF, 100 ng/mL TGF-β1, 10 ng/mL insulin, 100 ng/mL selenium, 1.25 mg/mL bovine serum albumin (BSA), and 5.35 mg/mL linoleic acid. Limiting dilution. To obtain single cell-derived colonies, the initial cell-seeded and divided plates were serially diluted and seeded on to 96-well plates (Becton Dickinson) in expansion medium. Cells were allowed to adhere overnight and nonadherent cells were washed out with medium changes. Medium changes were carried out twice weekly thereafter. Expansion medium consists of Iscove modified Dulbecco medium (IMDM; Gibco, Grand Island, NY) supplemented with 10 ng/mL bFGF, 100 U penicillin, 1000 U streptomycin, 40 μg/mL proline (Sigma-Aldrich), 10 ng/mL TGF-β1, 10 ng/mL HGF, 0.5 mM dexamethasone, and 50 mg/mL ITS+ premix, for 14 days followed by maturation medium thereafter. Maturation medium consists of IMDM supplemented with 20 ng/mL HGF, 0.5 μM dexamethasone, and 50 mg/mL ITS+ premix. Medium changes were carried out twice weekly and hepatogenesis was assessed at time points as indicated by reverse transcription-polymerase chain reaction (RT-PCR).

Histologic, cytochemical, and immunocytochemical analysis

Cytochemical staining. For evaluation of mineralized matrix, cells were fixed with 4% formaldehyde and stained with 1% Alizarin-red S (Sigma-Aldrich) solution in water for 10 minutes. In addition, mineralized matrix was also evaluated by von Kossa staining using 1% silver nitrate (Sigma-Aldrich) under UV light for 45 minutes, followed by 3% sodium thiosulfate (Sigma-Aldrich) for 5 minutes, and then counterstained with van Gieson (Sigma-Aldrich) for 5 minutes. For oil-red O staining, cells were fixed with 4% formaldehyde, stained with oil-red O (Sigma-Aldrich) for 10 minutes, and then counterstained with Mayer hematoxylin (Sigma-Aldrich) for 1 minute.

Histologic analysis. Chondrogenic differentiation was evaluated after pellets were fixed in 4% formaldehyde, dehydrated in serial ethanol dilutions, and embedded in paraffin blocks. Blocks were cut and sections stained with safranin-O (Sigma-Aldrich).

Immunofluorescence. For staining of intracellular proteins, cells were fixed with 4% formaldehyde at 4°C and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) for 10 minutes. 15 μg/mL mouse primary antibodies against human osteopontin (1:50), human MAP2 (1:50), human GFAP (1:50), or human albumin (1:50) for 1 hour, followed by fluorescein- or phycoerythrin-conjugated goat antiserum IgG secondary antibody for 1 hour. Between incubations, slides and dishes were washed with PBS.

Flow cytometry. For cell surface antigen phenotyping, fifth- to seventh-passage cells were detached and stained with fluorescein- or phycoerythrin-conjugated antibodies and analyzed with FACSCalibur (Becton Dickinson).

Total RNA isolation and RT-PCR

RNA was extracted from 3 to 30 × 10^6 MSCs, differentiating cells, or differentiated cells using RNEasy (Qiagen, Stanhope, Valencia, CA) per the manufacturer’s instructions. The mRNA was reverse transcribed to cDNA using Advantage RT-for-PCR (Clontech, Palo Alto, CA) per the manufacturer’s instructions. cDNA was amplified using an ABI GeneAmp PCR System 2400 (Perkin Elmer Applied Biosystems, Boston, MA) at 94°C for 40 seconds, 56°C for 50 seconds, and 72°C for 60 seconds for 35 cycles, after initial denaturation at 94°C for 5 minutes. Primers used for amplification are listed in Table 1.
Calcium imaging

For measurement of \([\text{Ca}^{2+}]_i\), differentiated cells were incubated in serum-free differentiation medium with 5 \(\mu\)M fura-2 AM for 1 hour at 37°C. Cells were then washed 3 times with the serum-free differentiation medium and used for measurements. For fura-2 excitation, a λ DG4 system (Sutter, Novato, CA) was used, which was controlled by Metafluor software from Universal Imaging (Downingtown, PA). Ratiometric calcium estimates were made by using 10-nm wide filters centered on 340 and 380 nm (Chroma Technology, Brattleboro, VT), capturing the emitted light (485-540 nm) at each excitation wavelength for 300 ms through a ×20 objective (Zeiss Axiovert 200 microscope) and directing it to a cooled CCD camera (CoolsnapFx; Roper Scientific, Tucson, AZ). The ratio within each cell was computed from images obtained at 340 and 380 nm excitation wavelengths and subtracting the appropriate background fluorescence at each wavelength. Ratios were computed every second.

To stimulate the cell, a 3-μm glass micropipette loaded with HighK buffer (5 mM NaCl, 5 mM glucose, 10 mM HEPES [N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid], 1 mM MgCl2, 150 mM KCl, 2.2 mM CaCl2, pH 7.3) was positioned at 10 μm from the recorded cell. The HighK buffer was puffed onto the cell for 3 seconds at 5 psi under the control of Picospitzer III (Parker Instrument, Parker Hannifin, Fairfield, NJ).

Results

Immunophenotypic characterization of UCB-derived MSCs

Fibroblast-like, rapidly diving cells (Figure 1) arising from limiting dilution were extensively expanded, and characterization by flow cytometry revealed that the cells isolated by the described method were negative for CD3 (T3), CD7 (gp40), CD19 (B4), CD34 (gp105-120), CD45 (leukocyte common antigen), CD117 (c-kit), CD133 (AC133), and CD135 (Flt-3), indicating these cells are not of hematopoietic origin (Figure 2A). Cells were also negative for matrix receptors CD31 (PECAM-1), CD62L (L-selectin), and CD62P (P-selectin), and negative for CD33 (P67), CD90 (Thy-1), and HLA-DR (Figure 2A). UCB-derived cells were found to be positive for integrins CD29 (β1-integrin), CD49b (α5-integrin), CD49d (α6-integrin), and CD51 (α2-integrin); positive for matrix receptors CD44 (hyaluronate receptor), CD58 (LFA-3), and CD105 (endoglin); and positive HLA-ABC (Figure 2A), which are consistent with the findings for bone marrow MSCs in the literature.9 In addition, the markers for human MSCs, SH-2, SH-3, and SH-4 (Figure 2B), were strongly positive.

In vitro differentiation of osteocytes, chondrocytes, and adipocytes from UCB-derived MSCs

To investigate the osteogenic potential of the UCB-derived cells, fifth- to seventh-passage cells were plated at a density of 3 × 10^3 cells/cm^2 and cultured under conditions appropriate for inducing differentiation for each lineage. When induced to differentiate under serum-free osteogenic conditions, the spindle shape of UCB-derived cells flattened and broadened with increasing time of induction (Figure 3A) and formed mineralized matrix as evidenced by Alizarin red staining (Figure 3B) and von Kossa staining (Figure 3C). The osteoblastic phenotype was also shown by the expression of marker genes osteopontin (hOSP) and osteocalcin (hOC), and by the gradual increase in type I collagen (hCol I) expression (Figure 3D). After 21 days of induction, cells were positive for immunofluorescence staining for osteopontin (Figure 3E). Immunofluorescence assays on undifferentiated cells stained negative for osteopontin (not shown).

The chondrogenic potential of UCB-derived cells was evaluated by culturing 1 × 10^6 cells under the pelleted micromass system in serum-free chondrogenic medium. After 3 weeks of differentiation the accumulation of sulfated proteoglycans was visualized by safranin-O staining (Figure 3F). Expression of mRNA of type II collagen (hCol II) and syndecan (hSDC) as well as perlecan (hPRLN), marker genes for chondrocytes, was detected by RT-PCR after 14 and 21 days of induction, and the expression of aggrecan (hAGCN) detected at 21 days after induction (Figure 3G). Immunohistochemical analysis for human type II collagen 21 days after induction was also positive (Figure 3H). To assess the adipogenic potential, fifth- to seventh-passage cells were plated at a density of 3 × 10^3 cells/cm^2 and cultured in adipogenic medium. Morphologic changes in cells as well as the formation of neutral lipid vacuoles were noticeable as early as 1 week after induction (Figure 3I) and visualized by staining with oil-red O (Figure 3J).
In vitro differentiation of neuroglial-like and hepatocyte-like cells from UCB-derived MSCs

Using the multistep induction method, fifth- to seventh-passage UCB-derived cells were seeded at a density of $3 \times 10^4$ cells/cm$^2$ and tested for their neurogenic potential. After 10 days of differentiation, 69% of cells in the flask acquired the morphology of neuroglial cells exhibiting a refractile cell body with extended neuritelike structures, and in areas of higher cell density, differentiated cells arranged into a networklike structure (Figure 4A); 17% of cells had partially acquired the morphology of neuroglial cells, and 13% of cells exhibited morphology resembling those of restricted precursors of the neuroectodermal lineage. RT-PCR analysis revealed expression of GFAP, a marker gene of glial cells, by day 6 and at day 10, whereas the expression of neuronal marker gene, MAP-2, was barely detectable at day 6 and had increased by day 10 (Figure 4B). In addition, 2% of cells stained positive for immunofluorescence assays against MAP-2 (Figure 4C) at 6 days after differentiation and increased to approximately 63% by day 10, whereas 11% stained positive against GFAP (Figure 4D) at 6 days after differentiation and increased to approximately 26% by day 10. Immunofluorescence assays on undifferentiated cells stained negative for MAP-2 and GFAP (not shown). After 12 days of differentiation neuronlike cells showed acute and significant elevation in $[\text{Ca}^{2+}]$, as determined by the change in ratio of fura-2 fluorescence, when challenged with a high concentration of extracellular potassium, whereas undifferentiated cells did not show significant changes in $[\text{Ca}^{2+}]$, (Figure 4E). The change in ratio of fura-2 fluorescence was 0.017 ± 0.002 for undifferentiated cells and 0.649 ± 0.066 for neuronlike cells (Figure 4F).

Schwartz et al$^{10}$ reported that a higher cell density favored hepatogenesis, whereas densities less than $12.5 \times 10^3$ failed to give rise to hepatocyte-like cells. To determine whether UCB-derived cells can differentiate into hepatocyte-like cells in vitro, cells were allowed to grow to 60% confluency prior to induction. The cuboidal morphology of hepatocyte-like cells was observed as early as 21 days after culturing under hepatogenic conditions and further matured by day 28 (Figure 4G) in the presence of oncostatin M. The expression of liver-associated genes was detected at the indicated time points (Figure 4H). As early as 1 week after induction more than 70% of cells stained positive for immunofluorescence assays against human albumin (Figure 4I).

After culturing under hepatogenic conditions for 6 weeks, cells exhibited the ability to take up low-density lipoprotein (LDL; Figure 4J). Undifferentiated cells were negative for albumin and did not show the ability to take up LDL (not shown).

Discussion

The morphology of these cells from UCB resembles that of MSCs isolated from the bone marrow. The self-renewal capacity of these cells is remarkable and is expected of multipotent stem cells. Flow cytometric analysis showed that these cells were negative for various lineage markers but positive for human MSC markers SH-2, SH-3, and SH-4, as well as various other integrins and matrix receptors, and is consistent with that reported in the literature for the bone marrow counterpart, indicating the MSC nature of these UCB-derived cells.

The capacity of UCB-derived cells to differentiate into osteoblasts that produce mineralized matrices, chondrocytes that produce type II collagen, and adipocytes that accumulate lipid vacuoles under in vitro conditions is consistent with that reported for bone marrow MSCs and was demonstrated by morphology, RT-PCR analysis, and histochemical, cytochemical, and immunocytochemical evaluations.

In addition to the potential of MSCs to differentiate into multiple lineages of the mesoderm, recent reports in the literature have indicated that bone marrow MSCs are capable of cell fates crossing germ layer boundaries.$^{11,12}$ The derivation of neuroglial cells of ectodermal origin$^{13}$ and hepatocyte-like cells of endodermal origin$^{14,15}$ from MSCs has been documented. However, skepticism has commonly been raised against such findings because these reports failed to show that differentiation into tissue lineages of mesodermal origin as well as nonmesodermal origin can be achieved from a single clonally expanded population of
MSCs. Instead, a plate-adhering population obtained from plating total mononuclear cells was used, raising the possibility that such a heterogeneous population may contain progenitors of all 3 germ layers and that different inductive conditions simply favored the proliferation and differentiation of different progenitor cells, hence leading to misinterpretation. On the contrary, we further investigated the potential of UCB-derived cells to differentiate into neuroglial-like and hepatocyte-like cells using the same clonally expanded population.

Under neurogenic conditions, UCB-derived cells exhibited the morphology and expressed genes specific of neuroglial cells, which was further confirmed by immunofluorescence assays for neuroglial-specific proteins. Undifferentiated cells did not express neuroglial marker genes and did not stain positive for neuroglial-specific proteins by immunofluorescence assays.

Voltage-sensitive ion channels are critical to the functioning of neuronal cells. When the concentration of extracellular potassium is elevated the membrane potential of neuronal cells is depolarized and the voltage-sensitive calcium channels open to allow an influx of calcium ions. When the differentiated cells were exposed to a high concentration of extracellular potassium a significant elevation in $[\text{Ca}^{2+}]$ was observed, indicative of a resting membrane potential and the presence of voltage-sensitive calcium channels on the membrane. Undifferentiated cells did not show any significant change in $[\text{Ca}^{2+}]$, under the same conditions.

When UCB-derived cells were cultured under hepatogenic conditions all cells acquired a cuboidal morphology as opposed to the fibroblast-like morphology of undifferentiated cells. Under hepatic conditions low-level expression of $\alpha$-fetoprotein, an early developmental marker gene of hepatoblasts, was detectable by day 7 and remained detectable up to day 35. Expression of cytokeratin-18 and albumin was detected at all time points, and the expression of tyrosine aminotransferase, a late marker gene of hepatocytes, was detected by day 14 and increased with time of differentiation. Furthermore, by day 7, differentiated cells stained positive for albumin by immunofluorescence analysis. Undifferentiated cells did not express $\alpha$-fetoprotein or tyrosine aminotransferase, but expressed low levels of albumin and cytokeratin-18. However, undifferentiated cells were negative for albumin by immunofluorescence analysis. After 6 weeks of differentiation the hepatocyte-like cells demonstrated the ability to take up LDL, a function characteristic of hepatocytes, whereas undifferentiated cells failed to take up LDL.

MSCs have been studied extensively over the past 3 decades and numerous independent research groups worldwide have successfully isolated MSCs from a variety of sources, most commonly, from the bone marrow. However, controversy exists over whether such stem cells are present in UCB and, to date, little evidence in the literature substantiates the existence of such cells in UCB. Erices et al and Goodwin et al independently published...
the successful isolation of progenitor cells of mesenchymal origin from UCB, whereas Mareschi et al. concluded against such findings.

By definition, MSCs are fibroblast-like in morphology, self-renewable, and capable of differentiating into, at a minimum, 3 connective tissue lineages of the mesoderm, namely, bone, cartilage, and fat. We have shown in the present study that a plate-adhering population of fibroblast-like cells isolated from UCB, indeed, can be extensively clonally expanded in vitro while retaining the potential to differentiate, under in vitro conditions, into multiple lineages of the mesoderm.

Erices et al. reported that UCB-derived mononuclear cells gave rise to 2 adherent cell types, one of which expressed MSC-related surface antigens. However, the multilineage differentiation ability of their cells was not shown in that study. Although Goodwin et al. have shown that their UCB-derived progenitor cells can differentiate into osteoblasts and adipocytes, the ability to differentiate into chondrocytes was not shown. Further, the expression of MSC-specific antigens such as SH-2, SH-3, and SH-4 was not demonstrated. In addition, although Goodwin et al. showed that their cells also expressed neural markers, a heterogeneous plate-adhering population from total mononuclear cells was used in their study, and the presence of neural progenitors in UCB has been documented. In this study, we have demonstrated that our relatively more homogeneous UCB-derived cells can differentiate into all 3 mesodermal lineages and, furthermore, the abilities to differentiate into neuroglial-like and hepatocyte-like cells were also evidenced. As a result of the broad differentiation potential of these cells the term MSC may seem somewhat inappropriate but, due to the similarities between these UCB-derived cells and the bone marrow MSCs, this nomenclature was adopted for the purpose of comparison, while research efforts are ongoing to fully characterize the differentiation potential and to determine a more appropriate terminology for these cells.

This report puts to rest the controversies and substantiates that UCB does contain MSCs. With the approach reported in this study, it is possible to obtain single cell-derived, clonally expanded MSCs from UCB with remarkable potential to differentiate into multiple lineages of mesodermal and nonmesodermal origin. With this technique, the application of UCB can be further extended and can be used as an alternative source of MSCs to bone marrow.
References


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