Isolation and characterization of human umbilical cord mesenchymal stem cells with hematopoiesis-supportive function and other potentials

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Background and Objectives. Adult bone marrow (BM) is the major source of mesenchymal stem cells (MSC) for cell therapy. However, aspiration of BM involves invasive procedures. We isolated MSC from human full term umbilical cord tissues (UC). The biological characteristics of MSC derived from UC (UC-MSC) were further determined and compared with normal adult bone marrow-derived MSC (BM-MSC).

Design and Methods. MSC were isolated from UC by enzyme digestion and cultured in appropriate growth medium. The isolation efficiency, cell yield, colony-forming unit-fibroblast (CFU-F) frequency, growth kinetics, phenotypic characteristics, multi-lineage differentiation capacity, cytokine spectrum as well as hematopoiesis–supportive function of UC-MSC were determined and compared with those of BM-MSC.

Results. MSC were successfully isolated from all 36 UC and six BM samples we collected for this study. The mean number of nucleated cells isolated from UC was 1×10^6/cm and the yield of adherent cells was 8.6×10^5/cm. UC-MSC shared most of the characteristic of BM-MSC, including fibroblastic-like morphology, immunophenotype, cell cycle status, adipogenic and osteogenic differentiation potentials, and hematopoiesis-supportive function. The CFU-F frequency was higher in UC nucleated cells (1:1609±0.18) than in BM nucleated cells (1: 35700±0.01) ($p<0.05$). Furthermore, in comparison with BM-MSC, the UC-MSC had a higher proliferation capacity and lower levels of expression of CD106 and HLA-ABC ($p<0.05$). Immunofluoresent and western blot assays revealed that UC-MSC had a higher percentage of neuron specific enolase-positive cells than had BM-MSC after neuronal induction. Finally, reverse transcriptase polymerase chain reaction analysis showed that UC-MSC had a cytokine spectrum very similar to that of BM-MSC, including expression of the mRNA of stem cell factor, leukemia inhibitor factor, macrophage-colony stimulating factor, Flt3-ligand, interleukin-6, vascular endothelial growth factor and stromal-derived factor-1, but UC-MCS additionally expressed mRNA of granulocyte macrophage and granulocyte colony-stimulating factors. After co-culture with $CD34^+$ cord blood cells for 5 weeks, no significant difference in colony-forming cells was observed between the $CD34^+$ cells/UC-MSC and $CD34^+$ cells/BM-MSC co-cultures ($p>0.05$).

Interpretation and Conclusions. We have established a protocol to isolate abundant MSC from human umbilical cords with a 100% success rate. The comparative study indicates that UC is an excellent alternative to BM as a source of MSC for cell therapies.

Key words: mesenchymal stem cells, umbilical cord, cord blood stem cells, hematopoiesis, cytokine.

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Mesenchymal stem cells (MSC) are of great therapeutic potential due to their capacity of self-renewal and multilineage differentiation.[1-5] They support hematopoiesis and enhance the engraftment of hematopoietic stem cells after co-transplantation.[6,7] Experimental and clinical data also demonstrated an immunoregulatory function of bone marrow BM-derived MSC (BM-MSC), which may contribute to the reduction of the incidence of graft-versus-host disease following hematopoietic stem cell transplantation.[8,9] Currently, bone marrow (BM) represents the major source of MSC for cell therapy. However, aspiration of BM involves invasive procedures, and the frequency and differentiation potential of BM-MSC decrease significantly with age.[10] Therefore, the search for alternative sources of MSC is of significant value. It has been reported that MSC could be isolated from various tissues, including periosteum, trabecular bone, adipose tissue, synovium, skeletal muscle, deciduous teeth, fetal pancreas, lung, liver, amniotic fluid, cord blood and umbilical cord tissues (UC).[11-15] Among those, cord blood and UC may be ideal sources due to their accessibility, painless procedures to donors, promising sources for autologous cell therapy and lower risk of viral contamination. However, the data on the isolation of cord blood-derived MSC are controversial.[14,16] In addition, the process of isolation of MSC is at the expense of losing hematopoietic stem cells in cord blood. Thus, UC should be focused on as an alternative source of MSC.

The UC contains two arteries and one vein, which are surrounded by Wharton's jelly. The cord is covered by an epithelium which is derived from the developing amnion. Romanov and his colleagues isolated MSC from the endothelial and subendothelial layers of the UC vein.15 However, the success rate of isolation, which is one of the most

important factors regarding the clinical use, was not described. We attempted to isolate MSC according to the protocol described in the report, but the success rate was quite low (30%, three of ten UC) in our hands. This might be due to the low frequency of MSC in endothelial and subendothelial layers of the UC vein. In view of recent data showing that sections of UC, such as Wharton's jelly17 and perivascular tissue,18 contain MSC, we reasoned that large amounts of MSC could be isolated from the whole UC without tissue dissection. In this study, we established a protocol to isolate MSC from UC, and carefully analyzed the morphological, phenotypic and functional characteristics of the UC-MSC in comparison with BM-MSC.

Design and Methods

Isolation and cell culture

Umbilical cords (n=36; gestational ages, 39-40 weeks) were obtained from local maternity hospitals after normal deliveries. Tissue collection for research was approved by the institutional review board of the Chinese Academy of Medical Science and Peking Union Medical College. After having been minced into 1-2 mm^3 fragments, UC were incubated with 0.075% collagenase type II (Sigma, St Louis, MO, USA) for 30 min and then 0.125% trypsin (Gibco, Grand Island, NY, USA) for 30 min with gentle agitation at 37°C. The digested mixture was then passed through a 100 μm filter to obtain cell suspensions. Cells were plated at a density of 1×10^6 cells/cm^2 in non-coated T-25 or T-75 cell culture flasks (Beckon Dickinson, San José, CA, USA). Growth medium (UC-GM) consisted of Dulbecco's modified Eagle's medium with low glucose (DMEM-LG; Gibco) and 5% fetal bovine serum (FBS; HyClone, Logan, UT, USA), supplemented with 10 ng/mL vascular endothelial growth factor (VEGF; Sigma), 10 ng/mL epidermal growth factor (EGF; Sigma) 100 U penicillin/streptomycin (Sigma), and 2 mM L-glutamine (Gibco). Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37C°. After 3 days of culture, the medium was replaced and non-adherent cells were removed. The medium was then changed twice weekly thereafter. Once 60%-80% confluence had been reached, adherent cells were replated at a density of 1×10^4/cm^2 in UC-GM for expansion.

Human adult BM-MSC were isolated by bone marrow aspirates from the iliac crest of six normal healthy volunteers (ages, from 20 to 35 years) after informed consent. The BM-MSC were prepared as described previously with slight modification.[19] Briefly, mononuclear cells were collected by gradient centrifugation and seeded at a density of 1×10^6 cells/cm^2 in growth medium containing DMEM-LG (Gibco) and 10% FBS (HyClone) (BM-GM). The non-adherent cells were removed after 3 days. The medium was changed every 3 to 4 days thereafter. At 60% to 80% confluence, the cells were replated at a density of 1×10^4/cm^2 in BM-GM for expansion.

Table 1. Primers for RT-PCR.

	Primers	Size
Lipoproteinlipase	S 5'-ATGGAGAGCAAAGCCCTGCTC-3' A 5'-TACAGGGCGGCCACAAGTTTT-3'	298bp
Osteopontin	S: 5'-CTAGGCATCACCTGTGCCATACC-3' A: 5'-CAG TGACCAGTTCATCAGATTCATC-3'	330bp
SCF	S: 5'-CTCCTATTTAATCCTCTCGTC -3' A: 5'-TACTACCATCTCGCTTATCCA -3'	177bp
LIF	S: 5'-AACAACCTCATGAACCAGATCAGGAGC-3' A: 5'-ATCCTTACCCGAGGTGCAGGGCCGTAGC-3'	405bp
FL	S:5'-AAATCCGTGAGCTGTCTG-3' A:5'-GTCCAGGCTATACATCCTC-3'	708bp
M-CSF	S:5'-TGAGAGGCAGTCCGAGGGAT-3' A:5'-GAATTCCCTCTACACTGGCA -3'	231bp
IL-3	S:5'-CAAGCTCCCATGACCCAGAC-3' A:5'-AGATCGCGAGGCTCAAAG-3'	400bp
IL-6	S:5'-GTAGCCGCCCCACACAGACAGCC-3' A:5'-GCCATCTTTGGAAGGTTCAGG-3'	173bp
GM-CSF	S:5'-GTCTCCTGAACCTGAGTAGAGACA-3' A:5'-AAGGGGATGACAAGCAGAAAGTCC -3'	286bp
G-CSF	S:5'-AGCTTCCTGCTCAAGTGCTTAGAG-3' A:5'-TTCTTCCATCTGCTGCCAGATGGT-3'	335bp
SDF-1	S:5'-CCCTTCAGATTGTAGCCCGG-3' A:5'-CGATCCCAGATCAATGTGCC-3'	251bp
VEGF	S:5'-TCGGGCCTCCGAAACCATGA-3' A:5'-CCTGGTGAGAGATCTGGTTC-3'	516bp 648bp
β2-MG	S: 5'-TCTGGCCTTGAGGCTATCCAGCGT-3' A: 5'-GTGGTTCACACGGCAGGCATACTC-3'	267bp

Immunophenotype analysis using FACS

Cells from UC (n=10, P2 to P6) and BM (n=6, P2 to P6) were stained with phycoerythrin (PE)-conjugated antibodies against CD13, CD14, CD29, CD31, CD34, CD38, CD45, SH3 (CD73), CD166, HLA-ABC and HLA- DR, or fluorescein isothiocyanate (FITC)-conjugated antibodies against CD44, CD105 (SH2). Mouse isotypic antibodies served as the control. All the antibodies were purchased from Becton Dickinson (San Diego, CA, USA). Cells were stained in single label and then analyzed by flow cytometry with a FACScan (Becton Dickinson).

Colony-forming unit-fibroblast (CFU-F) assay

The frequency of CFU-F was measured using the method of Castro-Malaspina with slight modification.[20] In brief, 1×10^6 nucleated cells, isolated from UC (n=16) or BM (n=6), were seeded in UC-GM or BM-GM in T-25 flasks, and incubated in a humidified atmosphere with 5% CO_2 at 37°C. The medium was completely renewed every 3 days. The fibroblast colonies were counted on day 10 of culture. Cell clusters containing > 50 cells were scored as CFU-F colonies.

CFU-F in limiting dilution assay

The frequency of CFU-F was further determined by means of a limiting dilution assay. The nucleated cells

from UC (n=16) or BM (n=6) were seeded in UC or BM-GM in six-well plates in six dilution steps. UC: 1×10^5 /$\times10^4$/2.5×10^4/1.25×10^4/6.25×10^3/3.125×10^3 cells/well; BM: 2.0×10^6/1.0×10^6/5×10^5/2.5×10^5/1.25×10^5/6.25×10^4 cells/well. The culture medium was changed every 3 days. Colonies containing 50 cells or more were counted on day 10 of culture. The frequency of colonies was evaluated by means of the Poisson statistic.[21]

Proliferation studies

To study the doubling time, cells from UC (n=10) and BM (n=6) were seeded in six T-25 flasks. The cells from one flask were harvested daily and enumerated for six consecutive days. Three sets of cultures were done. The mean of the counts was calculated and plotted against culture time to generate a growth curve. The mean doubling time was obtained by the formula: $TD=t_plg2/$ (lgNt-lgN0). N0: the inoculum cell number; Nt is the cell harvest number and t is the time of the culture (in hours). The doubling time of cells from P2 to P10 was determined.

Cell cycle analysis

Adherent cells from UC (n=8, P2 to P6) and BM (n=6, P2 to P6) were permeabilized with 70% alcohol, followed by incubation with 10 μg/mL RNase A (Sigma). Cells were then incubated with 50 μg/mL propidium iodide (PI, Sigma). DNA content was analyzed by FACScan flow cytometry (Becton Dickinson).

Differentiation studies

The differentiation potential of MSC was examined using P2 cells (UC, n=10; BM, n=6).

Osteogenic and adipogenic differentiation: cells were plated in six-well plates at a density of 3,000 cells/cm^2. Specific induction medium was added 24 hours later. The osteogenic induction medium consisted of DMEM-LG supplemented with 10% FBS, 10 mmol/L β-glycerophosphate, 100 nmol/L dexamethasone and 0.2 mmol/L ascorbic acid-2-phosphate. The adipogenic induction medium consisted of DMEM supplemented with 10% FBS, 1 μmol/L dexamethasone, 5 μg/mL insulin, 0.5 mmol/L isobutylmethylxanthine (IBMX), and 60 μmol/L indomethacin. Cells maintained in regular growth medium were regarded as the control. All the reagents for induction were purchased from Sigma. After 2 weeks of induction, the cells were stained using the von Kossa procedure or oil red solution to detect the presence of calcium deposition in osteocytes or neutral lipid vacuoles in adipocytes, respectively.[22,23] The osteogeneic specific gene, osteopontin (*OPN*), as well as the adipogenic specific gene, lipoprotein lipase (*LPL*), were further detected by reverse transcriptase polymerase chain reaction (RT-PCR) as described below. The appropriate primers are listed in Table 1.

Neural differentiation: cells were plated into six-well plates at a density of 3,000 cells/cm^2. Induction culture was performed based on the method of Lee *et al*.[24] Briefly, cells were treated with step 1 to 4 medium consecutively. Each step medium was used in the culture for 3 days and then changed to the next step medium. Step 1 medium consisted of Iscove's modified Dulbecco's medium (IMDM) supplemented with 5 ng/mL basic fibroblast growth factor (bFGF), 0.5 μmol/L retinoic acid , and 1 mmol/L 2-mercaptoethanol. Step 2 medium consisted of IMDM supplemented with 1 μmol/L cyclic adenosine monophosphate (cAMP). Step 3 medium consisted of IMDM supplemented with 10 μmol/L hydrocortisone and 1 mmol/L cAMP. Step 4 medium consisted of IMDM supplemented with 20 ng/mL acidic fibroblast growth factor (aFGF), 10 ng/mL sonic hedgehog (SHH), 10 ng/mL brain-derived growth factor (BDNF), 10 ng/mL nerve growth factor (NGF), 25 ng/mL vitronectin, 0.1 mmol/L IBMX, 10 μmol/L forskolin and 20 nmol/L phorbol myristate acetate. Cells kept in regular growth medium were regarded as the control. IMDM was purchased from Gibco. bFGF, SHH and BDNF were purchased from R&D (Minneapolis, MN, USA). All the other reagents were purchased from Sigma. Neurogenesis was assessed by immunofluoresence and western blot on day 10 of induction as described below.

Immunofluoresent analysis of neural differentiation

On day 10 of neural induction, cells were fixed in 4% paraformaldehyde (Sigma) and permeabilized in 0.1% Triton X-100 (Sigma). After non-specific binding, the cells were incubated with primary antibodies against neuron-specific enolase (NSE), fibrillary acidic protein (GFAP) and myelin basic protein (MBP). The cells were then incubated with FITC-labeled appropriate secondary antibodies, followed by counterstaining with Hoechst 33258. All the antibodies were purchased from Chemicon (Temecula, CA, USA). Control experiments were performed with cells cultured in regular growth medium.

Western blot analysis of neural differentiation

The protein levels of neural differentiation were determined by western blot analyses using standard procedures.[25] Protein lysates were prepared from cells on day 10 of neural induction. Equal amounts (50μg) of protein from each culture were analyzed on 10% polyacrylamide gels and transferred to nitrocellulose membranes. Antibody against NSE was purchased from Chemicon (Temecula, CA, USA). Each membrane was stripped and reprobed with anti-β-actin antibody as a loading control. The measurements of protein expression were based on densitometry determination.

Reverse transcriptase-polymerase chain reaction

RT-PCR analyes for cytokines, lipoprotein lipase and osteopontin were performed. The cytokines included stem cell factor (SCF), leukemia inhibitor factor (LIF), Flt3-ligand (FL), macrophage-colony stimulating factor (M-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulation factor (G-CSF),

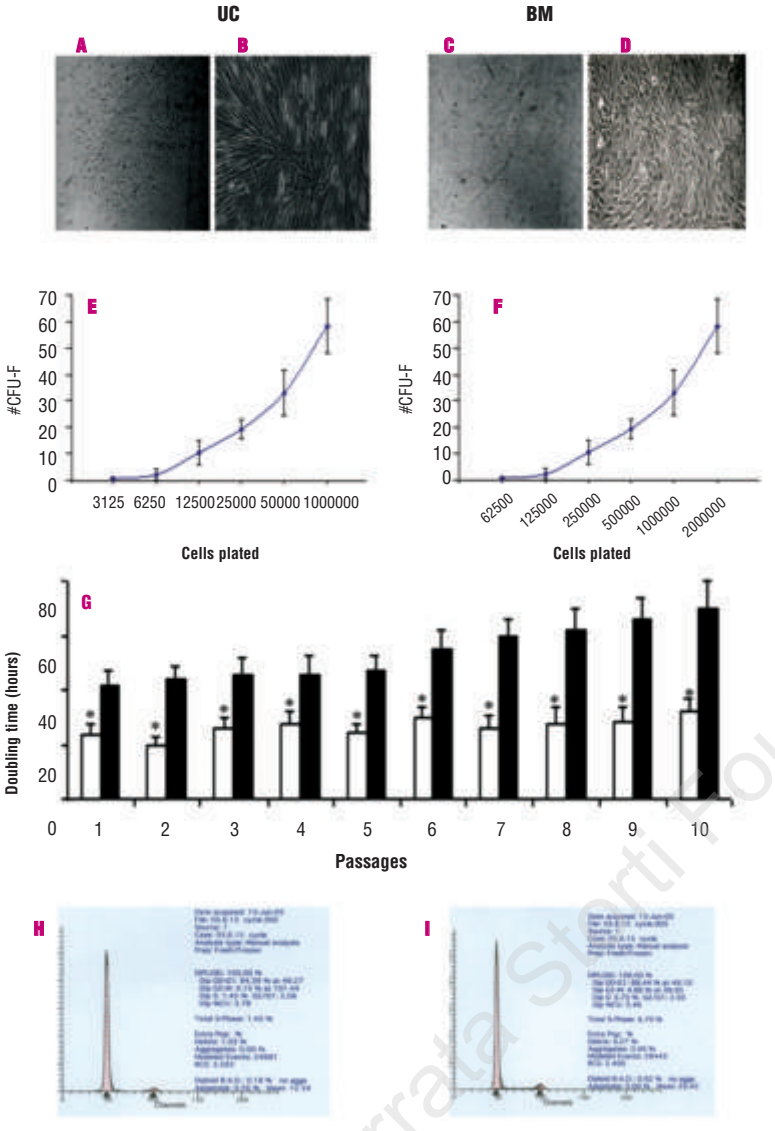


Figure 1. The morphology, colony-forming unit-fibroblast (CFU-F) and growth kinetics assay of umbilical cord (UC) or bone marrow (BM)-derived cells. (A-D) After the initiating plate on day 7 and day 14, adherent cells derived from UC (A-B) displayed a fibroblastic morphology similar to that of the adherent cells from BM (C-D) (magnification 100×). (E-F) CFU-F assay. The frequency of CFU-F in UC (E) or BM derived-nucleated cells (F) was determined by a limiting dilution assay. (G) Population doubling time of UC- (n=10, P1-P10) or BM -derived cells (n=6, P1-P10) was determined. The mean doubling time of cells derived from UC (□) was shorter than that of BM (■) during P1 to P10. The *p* value was analyzed by a two-tailed *t*-test (UC vs. BM). **p*<0.05 (H-I), representative cell cycle plots from UC (H) or BM-derived cells (I).

interleukin-3 (IL-3), interleukin-6 (IL-6), stromal derived factor-1 (SDF-1), and vascular endothelial growth factor (VEGF). Total RNA was extracted from 1×10^6 cells using trizol reagent according to the manufacturer's introduction. The cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase system (Gibco). PCR amplification was performed following standard procedures. RT-PCR of human β2-microglobulin (β2-MG) was performed as a control for cDNA production. The appropriate primers are listed in Table 1.

Hematopoietic assays

For the hematopoietic assays 2.0×10^5 adherent cells (UC: n=8, P3 to P10; BM: n=6, P3 to P 10) were irradiated with 3000cGy in 24-well plates. The following day, $CD34^+$ cells (2×10^4/well) were seeded on the irradiated layers in Myelocult medium (Myelocult™, HCC-5100, Stem Cell Technologies, Vancouver, BC, Canada). The co-cultures were maintained at 37°C with 5% CO_2 by weekly replacement of half the medium. After 5 weeks, cells were harvested and plated in standard methylcellulose culture, containing 100 U/mL IL-3, 200 U/mL of GM-CSF and 4 U/mL of erythropoietin (Methocult, Stem Cell Technologies), for colony-forming cell (CFC, i.e. BFU-E plus CFU-GM plus CFU-GEMM) assay. Cultures were incubated in a humidified 5% CO_2 atmosphere at 37°C. BFU-E, CFU-GM and CFU-GEMM were enumerated day 14 of culture as previously described.[26] $CD34^+$ cells were freshly purified from cord blood using a CD34/magnetic activated cell sorting (MACS) isolation kit according to the manufacturer's instructions (Miltenyi Biotech, Bergisch Gladbach, Germany). The cord blood samples were obtained from the allogeneic donors with the UC. The purity of $CD34^+$ cells was 95± 5% (range from 90~100%).

Statistical analysis

Data are presented as mean±SD. Comparisons of continuous variables between more than two groups were

Table 2. Cell yield and surface markers of UC and BM-derived cells.

No.	Size (cm)	UC Total nucleated cells ($\times 10^7$)	UC Nucleated cells/cm ($\times 10^6$)	UC Total adherent cells ($\times 10^7$)	UC Adherent cells/cm ($\times 10^5$)	No.	Volume (mL)	BM Total nucleated cells ($\times 10^7$)	BM Nucleated cells/mL ($\times 10^7$)	BM Total adherent cells ($\times 10^6$)	BM Adherent cells/mL ($\times 10^5$)
A											
1	20	3.4	1.7	2.0	9.9	1	1	5.5	5.5	7.8	7.8
2	25	1.8	0.7	1.3	5.2	2	5	23.0	4.6	39.5	7.9
3	30	4.0	1.3	2.8	9.2	3	1	8.7	8.7	9.8	9.8
4	40	4.8	1.2	3.6	9.1	4	10	32	3.2	65.0	6.5
5	20	1.5	0.8	1.4	6.9	5	6	32.4	5.4	49.8	8,3
6	45	4.4	0.9	3.8	8.5	6	5	33.5	6.7	46.0	9.2
7	15	3.0	2.0	1.5	9.8	7	3	20.7	6.9	25.5	8.5
8	25	4.6	1.8	2.5	10.0	8	5	19.5	3.9	46.5	9.3
9	33	3.4	1.1	2.9	8.7	Mean	5	21.9	5.5	42.7	8.4
10	18	1.9	1.1	1.6	9.0						
11	40	4.2	1.1	3.6	9.0						
12	28	2.2	0.8	1.9	6.9						
13	25	1.9	0.8	1.8	7.4						
14	30	2.9	1.0	2.4	8.1						
15	35	5.1	1.5	3.2	9.2						
16	42	5.3	1.3	3.8	9.1						
17	48	3.4	0.7	3.0	6.3						
18	24	2.7	1.1	2.1	8.8						
19	32	2.6	0.8	2.4	7.6						
20	16	1.4	0.9	1.3	8.2						
21	38	5.6	1.5	3.5	9.3						
22	42	5.5	1.3	3.9	9.2						
23	10	0.8	0.8	0.8	8.0						
24	16	1.5	1.0	1.5	9.4						
25	30	4.9	1.6	2.6	8.7						
26	25	2.6	1.0	1.7	6.9						
27	20	1.5	1.8	1.7	8.5						
28	36	3.6	1.0	3.1	8.5						
29	25	5.0	2.0	2.5	10.0						
30	38	3.5	0.9	3.0	8.0						
31	33	2.6	0.8	2.6	7.9						
32	29	2.1	0.7	1.7	6.0						
33	15	1.5	1.0	1.3	8.5						
34	38	3.7	1.0	3.2	8.5						
35	40	5.3	1.3	3.7	9.2						
36	28	4.3	1.5	2.7	9.8						
Mean	30	3.1	1.0	2.6	8.6						
p value			0.0002*		0.97**						

B Surface markers	UC (%) n=10	BM (%) n=6	p^ value
CD13	++++	++++	NS
CD14	–	–	NS
CD29	++++	++++	NS
CD31	–	–	NS
CD34	–	–	NS
CD38	–	–	NS
CD44	++++	++++	NS
CD45	–	–	NS
CD73	++++	++++	NS
CD90	++++	++++	NS
CD105	++++	++++	NS
CD106	+	+++	0.001
CD166	+++	+++	NS
HLA-ABC	+++	++++	0.004
HLA-DR	–	–	

*(A): Summary of the amount of tissue harvested and cell yields from the UC and BM. The number of total adherent cells was counted on day 14 in primary culture. The yield of adherent cells per cm of UC or per mL of BM was calculated by dividing the number of adherent cell by tissue mass. The p value was analyzed by two-tailed t-test. *p value was determined by the number of nucleated cells/cm of UC vs. that of BM. ** p value was determined by the number of adherent cells/cm of UC vs. that of BM. (B): Surface markers were detected by flow cytometric analysis. - negative <1%, + ¯++++ positive, + 1–25%, ++ 25%–50%, +++ 50%–75%, ++++ > 75%. p^ value analyzed by the two- tailed t–test (UC vs. BM). UC: umbilical cord; BM: bone marrow; NS: not significant.*

performed by a one-way ANOVA. If the *F* distribution was significant, a *t*-test was used to specify differences between groups. $p<0.05$ was considered statistically significant. The SPSS software package was used for the statistical tests.

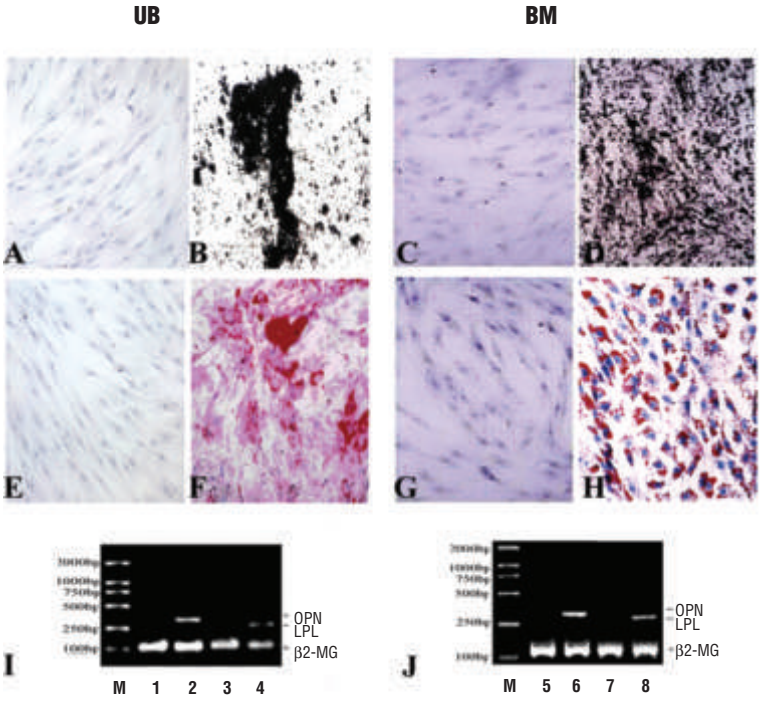


Figure 2. Adipogenic and osteogenic differentiation. (A-D) Osteogenic differentiation was assayed by the von Kossa procedure. No mineralized matrix formation was found in either UC- or BM-derived cells maintained in regular growth medium (A,C). Osteogenic differentiation was evidenced by the formation of mineralized matrix in both UC (B) and BM-derived cells (D) after osteogeneic induction. (E-H) Adipogenic differentiation was detected by oil red O staining. No lipid vacuoles were found in either UC- or BM-derived cells maintained in the regular medium (E,G). Adipogenic differentiation was evidenced by the formation of lipid vacuoles by oil-red O staining in UC- (F) and BM-derived cells (H) after adipogenic induction. All the representative samples are shown at 100× magnification. (I,J) Adipogenic and osteogenic differentiation of UC (I) and BM-derived cells (J) was further confirmed by the expression of adipocyte-specific genes (*LPL*) or bone-specific genes (*OPN*) by RT-PCR. No expression of *OPN* was found in UC- or BM-derived cells maintained in the regular medium (lanes 1 and 5). After osteogenic induction, the UC- and BM-derived cells expressed the mRNA of OPN (lanes 2 and 6). No expression of *LPL* was found in UC- or BM-derived cells maintained in the regular medium (lanes 3 and 7). After adipogenic induction, the UC- and BM-derived cells expressed the mRNA of LPL (lanes 4 and 8). M: marker, *LPL*: lipoprotein lipase, *OPN*: osteopontin.

Results

Isolation of adherent cells

After enzyme digestion of UC fragments, cells were seeded in UC-GM at a density of $1.0\times10^6/cm^2$. Adherent cells with fibroblastic morphology could be observed as early as 24 hours. The cells formed a monolayer of homogenous bipolar spindle-like cells with a whirlpool-like array within 2 weeks (Figure 1 A-B). The morphology of the UC-derived cells was consistent with that of BM-derived cells (Figure 1 C-D). Fibroblastic-like cells were successfully isolated from all the 36 UC and six BM samples. This represented 100% harvesting efficiency. Although the mean number of nucleated cells of UC was significantly lower than that of BM (1×10^6/cm vs. 5.5×10^7/mL, p=0.0002), no significant difference in the yields of adherent cells was observed (8.6×10^5/cm vs. 8.4×10^5/mL, p=0.97). The mean size of UC was 30 cm (range 10~48 cm) and 2.6×10^7 adherent cells (range 0.8×10^7~3.7×10^7) were obtained from UC after the primary culture. Table 2A provides the quantitative parameters concerning the isolation of adherent cells from UC and BM.

CFU-F frequency and growth characteristics

The CFU-F assay was based on Castro-Malaspina's method.[20] The mean of CFU-F colonies per 1×10^6 nucleated cells was significantly higher in UC (800, range 300-2000) than in BM (36, range 16-64). Figure 1 (E-F) shows the CFU-F frequency determined by limiting dilution assay, which further confirmed a higher frequency of CFU-F in UC (1:1609±0.18) than in BM (1:35700±0.01). Figure 1E shows that the frequency of CFU-F increased cell-seeding densities for both UC and BM-derived cells. Figure 1G illustrates that the population doubling time of UC-derived cells was shorter than that of BM-derived cells through P1 to P10 (p<0.05). The mean doubling time of P1 cells was 24 h and remained approximately constant until P10. The UC-derived cells could be readily expanded *in vitro* by serial passage every 2-3 days for 30 passages, without visible changes in either the growth patterns or morphology, indicating the high proliferation potential of UC-derived cells. However, the mean doubling time of P1 cells of BM was 40 h, and increased notably after P6.

Cell cycle analysis demonstrated that more than 80% of UC-derived cells (P2 to P6) were in G_0-G_1 (median 90.34%; range, 82.44%~95.23%), whereas a small population of cells was engaged in proliferation (G_2-M phase: median 6.24%, range, 4.86%-10.21%; S phase: median 3.73%; range, 1.45-7.57%). No significant differences concerning the cell cycle analysis of the BM-derived cells were observed. Figure 1 (H-I) shows the representative cell cycle spots of UC and BM-derived cells.

Immunophenotype of UC-MSC

Table 2B shows that UC-derived cells shared most of their immunophenotype with BM-derived cells, including positivity for CD13, CD29, CD44, CD105 (SH2), CD106, CD73 (SH3), CD166 (stromal markers) and HLA-ABC, but

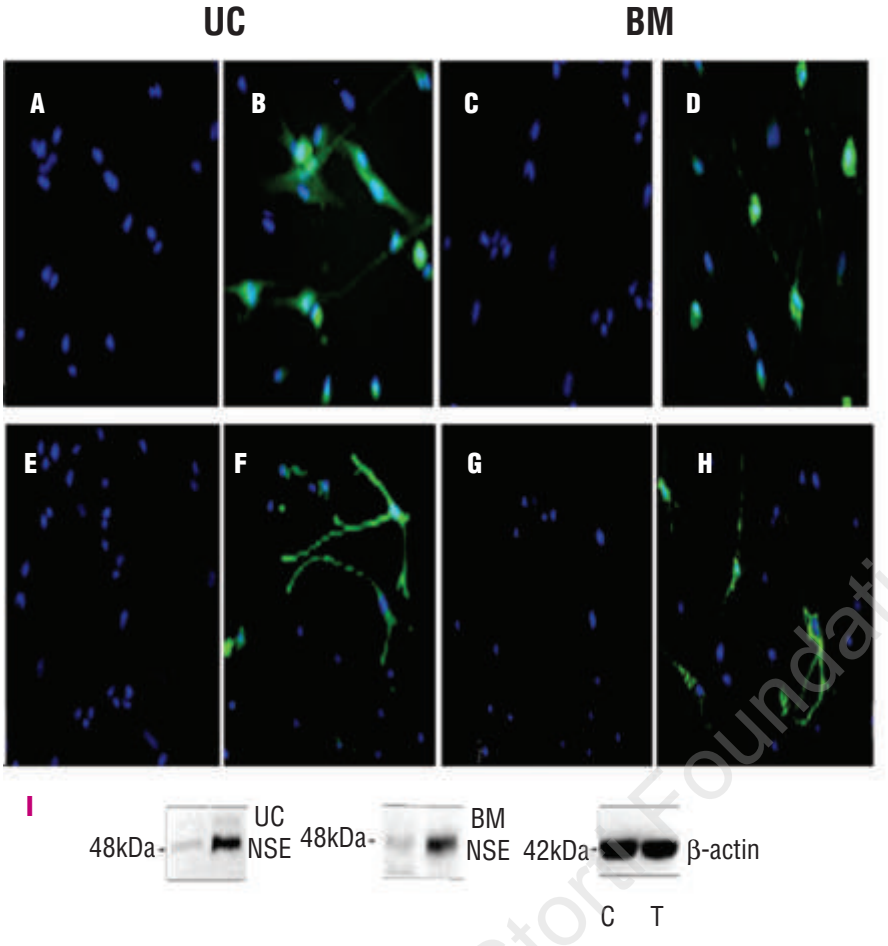
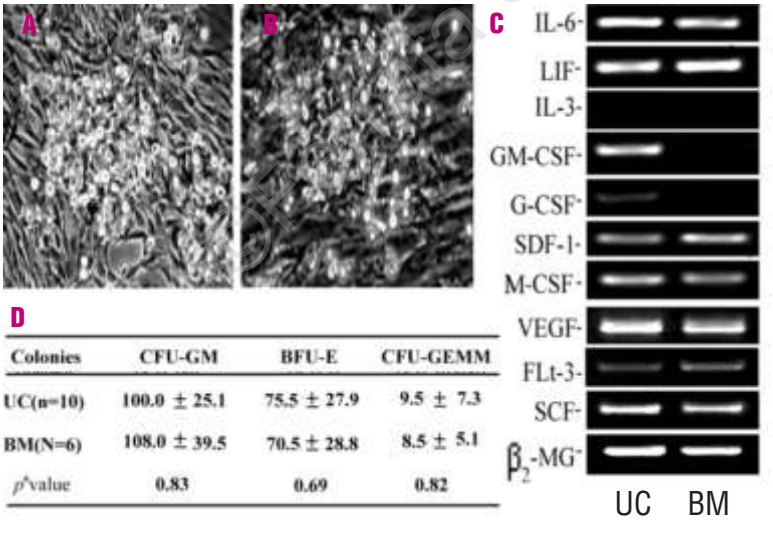


Figure 3. Neuronal differentiation of UC- and BM-derived cells was confirmed by immunofluoresence (A-H) and western blot (I). No cells positive for NSE or GFAP were found in UC (A,E) or BM-derived cells (C,G) that were maintained in regular medium. NSE-positive cells were found in UC- (B) and BM-derived cells (D) after neuronal induction. GFAP-positive cells were found in UC- (F) and BM-derived cells (H) after neuronal induction. The cell nucleus was stained by Hoechst 33258. All the representative samples are shown at 100× magnification. (I): Western blot illustrating the expression of NSE protein after neural induction. No expression of NSE was detected in cells maintained in regular growth medium. C: control cells were maintained in the regular growth medium. T: treated cells after neuronal induction. NSE: neuron specific enolase. GFAP: fibrillary acidic protein.



Colonies	CFU-GM	BFU-E	CFU-GEMM
UC(n=10)	100.0 ± 25.1	75.5 ± 27.9	9.5 ± 7.3
BM(N=6)	108.0 ± 39.5	70.5 ± 28.8	8.5 ± 5.1
*p**value	0.83	0.69	0.82

Figure 4. Hematopoiesis-supportive function assay. To investigate the hematopoiesis-supportive function, CD34$^+$ cells isolated from allogeneic cord blood were co-cultured with UC- or BM-derived feeder layer. (A-B) Typical cobblestone areas were seen in the CD34$^+$ cell/UC-MSC (A) and CD34$^+$ cell/BM-MSC (B) co-cultures (magnification 200× HP). (C) RT-PCR showed that UC-derived cells expressed the mRNA of SCF, LIF, M-CSF, Flt-3, IL-6, GM-CSF, G-CSF, SDF-1 and VEGF. The mRNA of IL-3 was not constitutively expressed by the UC-derived MSC. A similar cytokine spectrum was observed for the BM-derived cells. However, the BM-derived cells did not express either G-CSF or GM-CSF. (D) The co-cultures were maintained at 37°C in 5% CO_2 with weekly replacement of half the medium. After 5 weeks, cells were harvested and plated in standard methylcellulose culture for CFC (i.e. CFU-GM, BFU-E plus CFU-GEMM) assay. No differences were found in the numbers of CFU-GM, BFU-E or CFU-GEMM between UC- or BM-derived cells.

negativity for CD14, CD34, CD38, CD45 (hematopoietic markers), CD31 (endothelial cell marker) and HLA-DR. However, CD106 and HLA-ABC were significantly less expressed in UC-derived cells than in BM-derived cells (p=0.001 and p=0.004, respectively).

Differentiation capacities

Differentiation of MSC was assessed using P2 cells derived from UC (n=10) and BM (n=6). Cells kept in the regular growth medium served as the control.

Osteogenic differentiation

Osteogenic differentiation was detected by the calcification of the matrix (von Kossa) (Figure 2 A-D). All ten UC (100%) and five out of six BM (83%) samples formed mineralized matrix. RT-PCR further revealed the expression of an osteogenic specific gene, osteopontin, in the cells after osteogenic induction (Figure 2 I-J). No mineralized matrix or expression of osteogenic gene was observed in the cells kept in regular growth medium.

Adipogenic differentiation

Adipogenic differentiation was identified by oil red O staining (Figure 2 E-H). Ten out of ten UC (100%) and six out of six BM (100%) samples contained cells with an adipogenic phenotype. There was no significant difference in the percentage of positive cells between UC (69.4±4.5%) and BM-derived cells (57.3±9.6%) ($p>0.05$). RT-PCR further revealed the expression of an adipocytespecific gene, lipoprotein lipase, in the cells after adipogenic induction (Figure 2 I-J). The cells maintained in regular growth medium did not stain with oil red O and did not express lipoprotein lipase.

Neural differentiation

Neural differentiation was identified by immunofluoresent (Figure 3A-H) and western blot analysis (Figure 3I). After 10 days of induction, nine of ten of UC (90%) and five of six BM (83%) samples possessed cells with neural-like morphology that were positive for the special marker. The proportion of cells staining strongly for NSE (the neuronal specific marker) was higher in UC (60.8±3.9%) than in BM (40.3±6.2%) ($p<0.05$). No significant difference in GFAP (astrocyte marker) positive cells was observed between UC (4.2±1.3%) and BM (5.6±1.0%) ($p>0.05$). No MBP (oligodendrocyte marker) positive cells were observed in either UC or BM-derived cells after induction (*data not shown*). Western blot further confirmed the higher expression of NSE in UC than in BM-derived cells after the neural induction. Neither the immunofluorescent nor the western blot analysis showed expression of the specific marker for neural differentiation in cells maintained in regular growth medium.

Cytokine spectrum

In order to determine the cytokine spectrum, RT-PCR was performed in cells derived from UC (n=8, P3 to P10) and BM (n=6, P3 to P10). Figure 4C shows that UC-derived cells expressed the mRNA of SCF, LIF, M-CSF, Flt-3, IL-6, GM-CSF, G-CSF, SDF-1 and VEGF, but not that of IL-3. BM samples showed similar cytokine expression. However, the mRNA of G-CSF and GM-CSF was not detected in all the BM samples.

Hematopoietic assay

To assess the hematopoietic supportive function, allogeneic $CD34^+$ cells from cord bloods were plated on UC or BM-derived feeder layers. After culture in Myleocult medium for 5 weeks, the CFC (i.e. BFU-E plus CFU-GM plus CFU-GEMM) were enumerated. The number of CFC generated in 5-week old culture was representative of the number of long-term culture-initiating cells (LTC-IC). Figure 4A-B showed typical cobblestone areas in both the $CD34^+$ cells/UC-feeder layer and $CD34^+$ cells/BM-feeder layer co-cultures after 2 weeks of incubation. Figure 4D shows that there was no significant difference in numbers of BFU-E, CFU-GM or CFU-GEMM between $CD34^+$ cells/UC-MSC and $CD34^+$ cells/BM-MSC co-cultures ($p>0.05$). These results demonstrate that UC and BM-derived MSC both have similar hematopoiesis-supportive function.

Discussion

We successfully isolated fibroblastic-like cells from human full term UC and demonstrated their MSC characteristics by analyzing their morphology, immunophenotype, growth kinetics, multi-lineage differentiation potentials, cytokine spectrum and hematopoiesis-supportive function. Unlike previously reported methods,[15-18] in this study no section of UC tissue was discarded. The isolating frequency (36 of 36, 100%) was significantly higher than that from the vascular endothelia and subendothelia of UC according to the previous report.[15-17] The CFU-F frequency in UC nucleated cells obtained using our method was 1:1609 and was lower than that reported by Sarugaser *et al.* (1:333).[18] However, since the total number of nucleated cells harvested from UC ($0.7\text{-}2.0\times10^6$ cells/cm) using our method was much higher than that described previously ($2.5\text{-}25\times10^4$ cells/cm),[15-18] the absolute number of CFU-F obtained using our method was significantly greater that that obtained using the previously reported method.[18] These results thus suggest that the largest number of MSC can be isolated from the mixture of one organ without tissue dissection.

Not surprisingly, UC-MSC shared most of the characteristics with BM-MSC, including fibroblastic morphology, typical immunophenotypic markers, cell cycle status, adiopogeneic and osteogenic differentiation capacity, cytokine spectrum as well as hematopoiesis-supportive function. However, several differences were observed in this study. Firstly, the CFU-F frequency was significantly higher in UC-derived nucleated cells than in BM-derived nucleated cells. The CFU-F frequency of BM nucleated cells in our study is consistent with that in a previous study.[16] Since CFU-F represents the mesenchymal progenitor cell, our results suggest a higher frequency of MSC in the nucleated cells of UC than in those of BM.

Secondly, the proliferation analysis revealed that UC-MSC have a faster population doubling time. Such proliferation characteristics did not change even after 30 passages. In contrast, BM-MSC showed a significantly slow-

er population doubling time which became even longer after P6. These results indicate a higher proliferation capacity of UC-MSC in comparison with BM-MSC. In addition, UC-MSC showed lower expression of CD106 and HLA-DR in comparison with BM-MSC. The different expression of CD106 between UC-MSC and BM-MSC may represent a specific marker for identifying peripheral MSC from BM-MSC because low expression of CD106 has also been identified in adipocyte-derived MSC.[27] Furthermore, we were interested to note low expression of HLA-ABC on UC-MSC in addition to the absence of HLA-DR expression. This observation is consistent with the findings of Sarugaser *et al*.[18] Since HLA-ABC could be a hurdle for allogeneic cell therapies, the lower expression of HLA-ABC may favor the use of UC-MSC for allogeneic cell therapy.

Since both NSE- and GFAP-positive cells were observed after neural induction, this study suggests that UC-MSC have neural differentiation potential. This observation is in accordance with a previous report, describing that fibroblastic-like cells from Wharton's jelly could be differentiated into neural cells *in vitro*.[17] However, Sarugaser *et al*. described that the cells from perivascular umbilical cord could not be induced into neural cells.[18] This discrepancy may be due to the different methods of isolation or culture, by which the different subpopulation of MSC were isolated and expanded *in vitro*. Although the functional properties of differentiated neural cells of UC-MSC should be analyzed further in the future, our results suggest that UC-MSC have higher neural differentiation capacity than do BM-MSC. Since no MBP-positive cells were observed in either UC or BM-derived cells after induction, this neural induction protocol was most effective in neuronal differentiation.

UC-MSC, like BM-MSC, expressed IL-6, SCF, Flt-3, and M-CSF as detected by RT-PCR. Moreover, UC-MSC also expressed G-CSF and GM-CSF, which were not found in BM-MSC. These cytokines are associated with hematopoietic stem cell proliferation. Thus, the cytokine spectrum of UC-MSC could partly explain the hematopoiesis-supportive function of UC-MSC. SDF-1 and VEGF were expressed by both UC and BM-MSC. It has been known that enhancement of hematopoietic stem cell engraftment, produced by the addition of MSC, is partly due to MSC-produced cytokine and growth factors (*e.g.* SDF-1) capable of promoting the homing and/or expansion of the transplanted hematopoietic stem cells.[28,29] Our results suggest that UC-MSC could enhance the engraftment of cord blood transplants. In addition, VEGF has been demonstrated to enhance angiogenesis and tissue repair, thus contributing to the application of BM-MSC in stroke or myocardial infarction.[30] Our results further suggest the potential use of UC-MSC in neural or cardiovascular disorders.

In summary, the present study describes the isolation of abundant MSC from human full term umbilical cords without tissue dissection and demonstrates that UC-MSC share most of the characteristics with BM-MSC, including morphology, multi-lineage differentiation capacity and hematopoiesis-supportive function. In comparison with BM-MSC, UC-MSC have higher proliferation capacity and lower expression of CD106, HLA-ABC and HLA-DR, which should benefit their clinical use. Together with the distinct advantages of UC, such as accessibility, painless procedures to donors, possible source for autologous cell therapy and lower risk of viral contamination, we suggest that UC should be considered a promising alternative to BM as a source of MSC.

L-LL and Y-JL designed the research, performed the experiments, analyzed the data and wrote the manuscript; S-GY, Q-JZ, XW, WG, Z-BH, Z-SX, Y-XL and DL performed the experiments, collected and anlyzed thr data, Z-ZC participated in the design of the research and revised the manuscript, ZCH designed the research, analyzed the data and wrote the final version of the paper. The authors declare that they have no potential conflicts of interest. This work was supported by grants for projects 863 (2003AA205060, Z. C. Han) and 973 (001CB5101, Z. C. Han) from the Ministry of Science & Technology of China and a grant from the China National Foundation of Natural Science (30300186, 30570905, Y. J. Liu). We are grateful to Dr. Xuan Huang for his critical review of the manuscript.

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