Cytotherapy, 2015; 17: 1723–1731

International Society for Cellular Therapy ISCT®

CrossMark

DECIDUAL AND UMBILICAL CORD STROMAL CELLS

The effect of fibroblast growth factor on distinct differentiation potential of cord blood–derived unrestricted somatic stem cells and Wharton's jelly–derived mesenchymal stem/stromal cells

SEUNGOK LEE[1,2,*], BYUNG-JOON PARK[3,*], JI YEON KIM[1], DONGWOOK JEKARL[1,2], HYUN YOO CHOI[1], SEONG YEOUN LEE[1], MYUNGSHIN KIM[2], YONGGOO KIM[2] & MOON-SEO PARK[4]

[1]*Department of Laboratory Medicine, Incheon St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Incheon, South Korea,* [2]*Department of Laboratory Medicine, College of Medicine, The Catholic University of Korea, Seoul, South Korea,* [3]*Department of Obstetrics and Gynecology, Incheon St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Incheon, South Korea, and* [4]*Institute of Catholic Integrative Medicine, Incheon St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Incheon, South Korea*

Abstract

Background aims. Perinatal tissues are considered an attractive source of mesenchymal stem/stromal cells (MSCs) and have unique characteristics depending on their origin. In this study, we compared the basic characteristics of unrestricted somatic stem cells isolated from cord blood (CB-USSCs) and MSCs isolated from Wharton's jelly of umbilical cords (WJ-MSCs). We also evaluated the effect of basic fibroblast growth factor (bFGF) supplementation on the growth and differentiation of these cells. *Methods.* CB-USSCs and WJ-MSCs were isolated from the same individual (n = 6), and their morphology, cell surface antigens, proliferation, expression of stemness markers and adipogenic, osteogenic and chondrogenic differentiation potentials were evaluated. Their morphology, proliferation and differentiation potentials were then also compared in the presence of bFGF supplementation (10 ng/mL). *Results.* Overall, CB-USSCs expressed *DLK-1* and negative for all the *HOX* gene markers. The expression of cell surface antigen CD90, growth capacity and adipogenic differential potential of CB-USSCs were lower than those of WJ-MSCs. WJ-MSCs showed higher growth capacity, but the expression of CD73 and CD105 and their osteogenic differentiation potential were lower than those of CB-USSCs. The spindle morphology of both CB-USSCs and WJ-MSCs and the growth and adipogenic differentiation of CB-USSCs were improved by bFGF supplementation. However, the bFGF supplement did not have any positive effect on the tri-lineage differentiation potentials of WJ-MSCs. *Conclusions.* CB-USSCs and WJ-MSCs each had distinct characteristics including different growth capacity, distinguishable cell surface markers and distinct adipogenic and osteogenic potentials. bFGF supplementation improved the growth capacity and adipogenic differentiation of CB-USSCs.

Key Words: *basic fibroblast growth factor, mesenchymal stem/stromal cells, umbilical cord blood, unrestricted somatic stem cells, Wharton's jelly*

Introduction

Mesenchymal stem/stromal cells (MSCs) are multipotent precursor cells with self-renewal ability and have characteristics that vary depending on their origin in many adult and fetal tissues [1–3]. Ideally, for stem cells to be effective in regenerative medical applications, stem cells should be abundant and obtainable through a minimally invasive procedure to ensure donor safety [4].

Bone marrow (BM) is a standard source for the isolation of MSCs, but the procedure for BM harvest is highly invasive. Perinatal tissues, such as umbilical cord blood (CB), Wharton's jelly (WJ), amnion and

*These authors contributed equally to this work.

Correspondence: **Myungshin Kim**, MD, PhD, Department of Laboratory Medicine, College of Medicine, The Catholic University of Korea, 222 Banpo-daero, Secho-gu, Seoul 137-701, South Korea. E-mail: microkim@catholic.ac.kr

(Received 31 March 2015; accepted 18 September 2015)

ISSN 1465-3249
http://dx.doi.org/10.1016/j.jcyt.2015.09.007

chorion, are considered to be attractive sources of MSCs because they can be obtained by less invasive methods without harm to mothers or neonates [1,4–9].

To date, results from isolations of CB-MSCs from CB (CB-MSCs) have been inconsistent because of the low frequency and viability in CB after the donor's birth. Consequently, a large CB volume and a rapid isolation procedure after collection are needed to increase the success rate [5,8]. Therefore, MSCs derived from CB have not yet been widely used in clinical applications. CB-MSCs may also be obtained as functionally distinct heterogenous populations [5,10] in a nonselective manner by same isolation procedure. Recently, an unrestricted somatic stem cell population in CB (CB-USSCs) was identified with lower adipogenic differentiation potential that correlated with the expression of the δ-like 1 (*DLK-1*) gene [11]. These cells can be differentiated from the CB-MSCs population because of their lack of expression of the *HOX* genes, including *HOXA9*, *HOXB7*, *HOXC10* and *HOXD8* [10].

WJ is a connective tissue of the umbilical cord, and is histologically composed of myofibroblast-like stromal cells, collagen fibers, and proteoglycans [4,7,12]. Recently, WJ-MSCs have become known as an additional good source of stem cells. MSCs derived from WJ (WJ-MSCs) can be obtained more easily because the success rate is high (100%) and isolation is a less time-limited procedure than for CB-MSCs [3]. They are embryonic and somatic in origin, highly homogenous, have a greater expansion capacity than BM-MSCs and may have the potential to differentiate into several lineages, such as adipose cells, chondrocytes, osteoblasts, neuronal cells, hepatocyte-like cells and endothelial cells *in vitro* [3,4,7,13]. However, a minority of reports has questioned the stemness nature of WJ-MSCs because their differentiation potential into the tri-lineages has not been achieved *in vitro* [2]. However, WJ-MSC meet two requirements of the minimal criteria of the International Society for Cellular Therapy (2006), which state that MSCs should be plastic adherent when maintained in standard culture condition; express CD73, CD90 and CD105; lack expression of CD14, CD45 and CD34; and differentiate into adipoblasts, osteoblasts and chondrocytes *in vitro* [14].

For clinical applications using MSCs, growth factors such as basic fibroblast growth factor (bFGF), known as FGF-2, have been widely added to *in vitro* culture systems in stem cell research [15]. bFGF promotes population growth by increasing bFGF receptor signaling and affects differentiation potential into several lineages of adult stem cells, such as human BM and adipose MSCs [15–18]. However, not much is known about the effect of bFGF on the multi-potency of CB-USSCs or WJ-MSCs.

In this study, we first compared the basic characteristics of CB-USSCs and WJ-MSCs derived from the same individual (n = 6), with BM-MSCs. Second, we evaluated the effects of bFGF supplementation on morphology, proliferation and the adipogenic, osteogenic and chondrogenic differentiation potentials of CB-USSCs and WJ-MSCs.

Methods

Isolation of CB-USSCs

This study was performed in accordance with the ethical standards of the Declaration of Helsinki and was approved by the institutional review board of our institution. Healthy women with full-term pregnancies and without underlying disease or obstetrical complications and their uneventful newborn were included in the study. Three of six infants were male and the others female; all infants fell within normal range of parameters for healthy infants. Paired CB and umbilical cord were collected at delivery with informed consent of the mother at Incheon St. Mary's Hospital, the Catholic University of Korea.

CB-USSCs were isolated from CB using the same protocol used for harvesting CB-MSCs, according to the methods of Lee et al., Zhang et al. and Phuc et al. with modification [8,19,20]. The procedure was performed within 2 hours of CB collection. Briefly, CB was diluted in phosphate buffer saline (PBS) at a ratio of 1:1 and 15 mL of this diluted blood was gently loaded onto an equal amount of Ficoll Hypaque solution (1.077 g/mL, Sigma-Aldrich) in a 50-mL tube. After centrifugation at 1600 rpm for 20 min, mononuclear cells (MNCs) were obtained from the interphase layer and washed twice with PBS [20]. MNCs were counted by an automated cell analyzer XE-2100 (Sysmex). MNCs were plated in culture at a density of 1×10^6/cm^2 into six-well culture plates (BD falcon 353046) in culture medium and incubated at 37°C in a 5% CO_2 incubator [8]. Standard culture medium in this study consisted of α-modified minimum essential medium (α-MEM; Hyclone) and 20% fetal bovine serum (FBS; Hyclone), supplemented with 10 ng/mL bFGF (Gibco), 1% penicillin-streptomycin (Gibco) and Glutamax (1×; Gibco) [19]. Cells were allowed to adhere for 2 days and nonadherent cells were washed off with a medium change. The medium was changed twice weekly for 4 weeks. A colony of suspected MSCs was harvested using 0.25% trypsin-EDTA (1×; Gibco). For expansion, the cells were seeded at a density of 1×10^4/cm^2 [8].

Isolation of WJ-MSCs

MSCs were isolated from WJ according to the methods of Bakhshi et al. and Hsieh et al., with modification [13,21]. The procedure was performed within 24 h after

delivery. Briefly, a portion of the umbilical cord was cut into approximately 5-cm-long segments, and these were placed immediately into PBS. Each segment was dissected longitudinally. WJ were isolated from the segments and then digested with collagenase II (Gibco) for 1 h and trypsin/EDTA for 30 min. Cells were washed twice with PBS and cultured in a T25 flask (BD 353108) at 37°C in 5% CO_2 incubator. After approximately 4 days to 1 week, fibroblast-like adherent cells were detached using 0.25% trypsin/0.5 mmol/L EDTA and reseeded in fresh culture medium for further expansion [13].

MSCs from BM were obtained commercially from Texas A&M Institute for Regenerative Medicine. Expansion was performed according to the manufacturer's instructions.

Cell morphology and proliferation assay

The morphology of the pairs of second passage CB-USSCs and WJ-MSCs, maintained in standard culture medium either without or with bFGF supplementation (10 ng/mL), were examined under a phase contrast microscope by two laboratory experts. Cell proliferation rates of the pairs of CB-USSCs and WJ-MSCs (n = 6) and BM-MSCs (n = 2), without and with bFGF supplemented medium (10 ng/mL), were measured from days 1 to 9 after plating 8×10^2 cells in a 96-well plate. Cell viability was measured by the CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (Promega), according to the manufacturer's instructions [17]. All measurements were performed in triplicate.

Flow cytometry

Flow cytometry was performed for pairs of CB-USSCs and WJ-MSCs (n = 6), and BM-MSCs (n = 2) at the third or fourth passage. To evaluate cell-surface antigen expression, cell suspensions of 1×10^6 cells were incubated for 15 min at room temperature in the dark with phycoerythrin-conjugated monoclonal antibodies specific for the human markers associated with mesenchymal and hematopoietic lineages. The antibodies used were CD73, CD90, CD105, CD146, HLA DR, CD56 and CD45 (BD Biosciences). The samples were fixed using fixation buffer and analyzed on a FACSCanto II cytometer and the resulting data was processed using FACSDiva software (BD Biosciences).

Reverse transcriptase polymerase chain reaction for the identification of CB-USSCs

Reverse transcriptase polymerase chain reaction (PCR) for *HOX* markers (*HOXA9*, *HOXB7*, *HOXC10*, *HOXD8*) and *DLK-1* was carried out on CB-USSCs together with WJ-MSCs and BM-MSCs [10,11]. *HOX*-negative and *DLK*-positive cells were identified as CB-USSCs, according to the previous studies by Liedtke et al. and Klute et al. [10,11].

In vitro differentiation

MSCs from pairs of CB-USSCs and WJ-MSCs (n = 6), and BM-MSCs (n = 2) were differentiated into adipocytes, osteoblasts and chondrocytes after three passages, as previously described but with modification [3]. Briefly, for adipogenic and osteogenic differentiation, MSCs were seeded at a concentration of 4×10^4 for CB-USSCs, 3×10^4 for WJ-MSCs and 3.5×10^4 for BM-MSCs per 12-well plate, respectively, in the expansion medium without and with bFGF supplementation (10 ng/mL) for 3 days. Then the expansion medium was replaced by adipogenic or osteogenic induction medium (Gibco), without and with the bFGF supplement (10 ng/mL) for 3 weeks. Control cells were maintained in standard culture medium with 10% FBS during the same time period. After 3 weeks, cells were fixed in 4% formaldehyde. To assess adipogenic differentiation, lipid droplets of differentiated cells were stained with oil red O (Sigma-Aldrich). To assess osteogenic differentiation, cells were stained with alizarin red S (Sigma-Aldrich). For chondrogenic differentiation, 2.5×10^5 USSCs, WJ-MSCs or BM-MSCs per 15-mL conical tube (BD Biosciences) were centrifuged at 150 *g* for 5 min and subsequently cultivated in chondrogenic induction medium (Gibco) with or without the bFGF-supplement for 6 weeks. Control cells were maintained in standard culture medium with 10% FBS during the same time period. To assess chondrogenic differentiation, cell pellets were fixed in 4% formaldehyde and embedded in paraffin. Sections were stained with Alcian Blue (Sigma-Aldrich) and hematoxylin-eosin (H&E; Sigma-Aldrich).

Real-time quantitative PCR

Cell differentiation was also assessed using differentiation gene markers evaluated by real-time quantitative PCR, carried out with SYBR Green master mix (Takara Bio Inc.) on an ABI 7500 Detection System (Applied Biosystems) [10].

For adipogenic differentiation, fatty acid-binding protein 4 (*FABP4*), peroxisome proliferator-activated receptor gamma-2 (*PPARγ*) and lipoprotein lipase (*LPL*) markers were assessed at day 0 and 1 week [13]. For osteogenic differentiation, alkaline phosphatase (*ALP*), *collagen I* and runt-related transcription factor 2 (*Runx2*) markers were assessed at day 0 and 1 week [2,22]. For chondrogenic differentiation, SRY-Box 9 (*Sox-9*) was assessed at day 0 and 1 week [23].

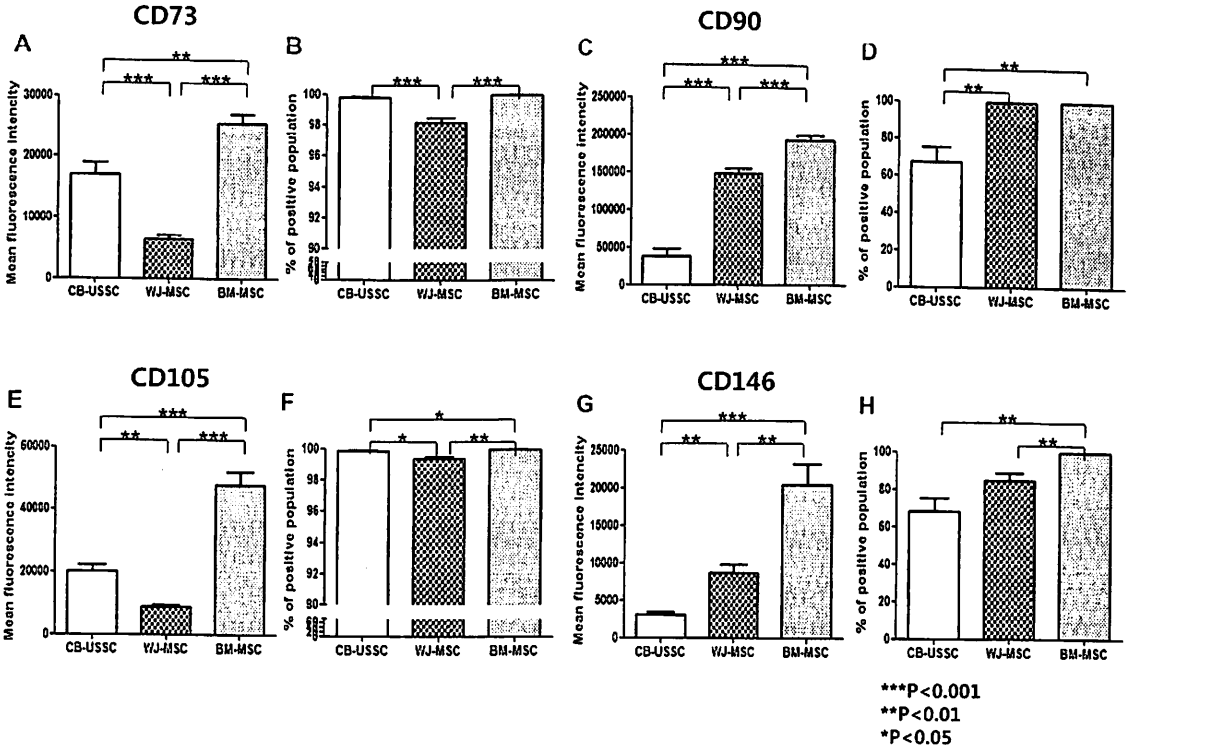

Figure 2. Flow cytometric evaluation of the expression of cell surface markers on CB-USSCs, WJ-MSCs, and BM-MSCs. Mean FI (A, C, E, G) and % of positive population (% $_{Pos}$, B, D, F, H) of CD73 (A), CD90 (B), CD105 (C) and CD146 (D) in CB-USSCs (n = 6) and WJ-MSCs (n = 6) compared with BM-MSCs (n = 2). All graphs show the mean ± SEM. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

The adipogenic differentiation potentials of CB-USSCs and WJ-MSCs

CB-USSCs and WJ-MSCs showed distinct adipogenic differentiation potential after 3 weeks of adipogenic induction. Fat vacuoles, characteristic of adipocyte-like cells, were most abundant in BM-MSCs (n = 2), followed by in WJ-MSCs (n = 6) in conditions without and with bFGF. The formation of fat vacuoles was not facilitated by the bFGF supplement (Figure 3). Adipogenesis was unsuccessful in CB-USSCs (n = 6) lacking bFGF. However, it was stimulated in four of six CB-USSCs by bFGF supplementation. Without bFGF, *FABP4*, *PPARγ* and *LPL* were expressed at low levels in CB-USSC (all *P*s < 0.001, all), compared with their expression levels in BM-MSCs. On the other hand, expression levels of *FABP4* and *LPL* were low in WJ-MSCs, compared with the levels expressed in BM-MSCs (all *P*s < 0.001). Of note, *PPARγ*, a prime inducer of adipogenesis [24], was highly expressed in WJ-MSCs when compared with CB-USSCs ($P < 0.001$ by paired *t*-test), indicating that *PPARγ* may have been a good adipogenic discrimination marker in this study (Figure 3, supplementary Table SIII).

The relative expression levels of adipogenic markers in CB-USSCs displayed a similar pattern 1 week after adipogenic induction with the bFGF supplement. However, *FABP4* and *PPARγ* were down-regulated in WJ-MSCs 1 week after adipogenic induction by bFGF supplementation ($P < 0.001$, $P = 0.001$, respectively), and all adipogenic markers (*FABP4*, *PPARγ* and *LPL*) were also down-regulated in BM-MSCs by the bFGF supplement ($P < 0.001$, $P = 0.008$, $P = 0.004$, respectively; supplementary Table SIV).

The osteogenic differentiation potentials of CB-USSCs and WJ-MSCs

After 3 weeks of osteogenic induction, CB-USSCs and WJ-MSCs showed distinct differentiation potentials. The degree of mineralization was assessed as ALP precipitates by alizarin red S staining and was found to be highest in five of six CB-USSCs. bFGF supplementation caused osteogenic differentiation to be slightly suppressed in five of six CB-USSCs and a more pronounced suppression was seen in bFGF-treated BM-MSCs (Figure 4). However, osteogenesis was unsuccessful in WJ-MSCs lacking bFGF, which was also not facilitated by the bFGF supplementation (n = 6). Under conditions lacking bFGF, the relative expression of all osteogenic markers (*ALP*, *Collagen I* and *Runx2*) was lower in CB-USSCs 1 week after induction, compared with those of BM-MSCs ($P = 0.03$, $P > 0.05$ and $P > 0.05$, respectively). These changes did not correlate with the morphology of the ALP precipitates visualized by alizarin red S staining (Figure 4,