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Differentiation of Umbilical Cord Lining Membrane-Derived Mesenchymal Stem Cells into Endothelial-Like Cells

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ABSTRACT

Background: Stem cell therapy for the treatment of vascular-related diseases through functional revascularization is one of the most important research areas in tissue engineering. The aim of this study was to investigate the *in vitro* differentiation of umbilical CL-MSC into endothelial lineage cells. **Methods**: In this study, isolated cells were characterized for expression of MSC-specific markers and osteogenic and adipogenic differentiation. They were induced to differentiate into endothelial-like cells and then examined for expression of the endothelial-specific markers, karyotype, and functional behavior of cells. **Results**: Isolated cells expressed MSC-specific markers and differentiated into adipocytes and osteoblasts. After endothelial differentiation, they expressed CD31, vWF, VE-cadherin, VEGFR1, and VEGFR2 at both mRNA and protein level, but their morphological changes were not apparent when compared with those of undifferentiated cells. There were no significant changes in karyotype of differentiated cells. Furthermore, angiogenesis assay and LDL uptake assay showed that differentiated cells were able to form the capillary-like structures and uptake LDL, respectively. **Conclusion**: The results indicated that umbilical CL-MSC could differentiate into functional endothelial-like cells. Also, they are suitable for basic and clinical studies to cure several vascular-related diseases. *Iran. Biomed. J. 18 (2): 67-75, 2014*

Keywords: Endothelial differentiation, Endothelial-like cells, Mesenchymal stem cells, Umbilical cord lining membrane

INTRODUCTION

One of the major difficulties in tissue engineering is vascularization that occurs by two distinct processes responsible for the development of blood vessels: vasculogenesis and angiogenesis [1]. Previous studies showed that endothelial cells were important participants in repair of damaged endothelium and the formation of new blood vessels [2], and might improve myocardial contractile function or perfusion in patients with vascular-related diseases, such as peripheral arterial disease or myocardial infarction disease [3, 4]. However, an obstacle to endothelial cell therapy is the difficulty in obtaining enough cells. Indeed, these cells could be expanded *in vitro* but gradually lose their proliferative potential and their functions. For this reason, it is necessary to find alternative cellular sources for autologous or allogeneic transplantation [5].

As an alternative, the utility of MSC has been hold great promise for treatment of the vascular-related diseases through functional revascularization. MSC, because of powerful *ex vivo* proliferation and multipotent differentiation, are an ideal candidate for cell-based therapy [6]. MSC expressed many angiogenic growth factors and might stimulate collateral vessel formation by paracrine mechanisms [7]. In addition, some of the injected MSC were incorporated in new blood vessels [8]. When cultured

*Corresponding Author; Tel.: (+84-8) 37309931; Fax: (+84-8) 37309963; E-mail: dcchung@hcmus.edu.vn or chungbiotech_uns @yahoo.com; **Abbreviations:** CL-MSC: umbilical cord lining membrane-derived MSC; HUVEC: human umbilical vein endothelial cells**;** LDL: low-density lipoprotein; MSC: Mesenchymal stem cells; VE-cadherin: vascular endothelial cadherin; VEGF: Vascular endothelial growth factor; VEGFR1: Vascular endothelial growth factor receptor 1; VEGFR2: Vascular endothelial growth factor receptor 2; vWF: von Willebrand factor; FITC: fluorescein isothiocyanate; PE: Phycoerythrin; DiI-Ac-LDL: 1,1′-dioctadecyl-1-3,3,3′,3′-tetramethylindocarbocyanine labeled acetylated low-density lipoprotein

in the presence of endothelial growth factor, MSC could express endothelial markers [9-11], that could differentiate into the endothelial-like cells. Another advantage of MSC, compared with blood-derived endothelial cells, was the greater number of cells that could be obtained from adult sources [12].

In recent years, many researchers have focused on various types of umbilical cord-derived MSC: umbilical cord matrix stem cells, umbilical cord perivascular stem cells, umbilical cord stroma cells, Wharton's jelly MSC and CL-MSC because of many advantages. They were more primitive MSC than those isolated from other tissue sources and possessed immunosuppressive properties, which have been widely used for transplantation. Furthermore, they had higher proliferation potential and differentiation capacity, non-invasive accessibility and lack of ethical controversy [13, 14]. However, very little data were reported on the endothelial potential of umbilical cord-MSC, except for some studies reported the efficiency of umbilical cord -MSC transplantation in ischemic mouse model through revascularization mechanism [11] or endothelial differentiation of Wharton's jelly-derived MSC in comparison with bone marrow-derived MSC [15]. Therefore, it is essential to make a more comprehensive assessment of the feasibility of using umbilical cord-MSC-derived endothelial cells in biomedical engineering.

In the study, we report that MSC isolated from cord lining membrane, a part of umbilical cord tissue, could differentiate into endothelial-like cells in an endothelial growth medium supplemented with VEGF. This information can help to develop cell-based therapeutics for various vascular-related diseases.

MATERIALS AND METHODS

Isolation and culture of CL-MSC. Umbilical cord samples were collected (with permission at Hung Vuong Hospital, Ho Chi Minh City (Viet Nam) from women in childbirth being negative for hepatitis B virus, hepatitis C virus, HIV with an informed consent. The samples were removed from residual blood and cut into 2-4 cm long pieces. Single pieces of umbilical cord was dissected and separated from the epithelium to expose the underlying Wharton's jelly. After removal of umbilical cord vein and arteries, the residual tissue pieces were incubated in DMEM at 37°C, 5% CO_2 for about 6 hours. Wharton's jelly absorbed DMEM; therefore, it can be distinguished from the membrane of umbilical cord. The Wharton's jelly was eliminated using razor blades, and pieces of cord lining membrane was rinsed and chopped into 2-3 mm^3 explants. They were then transferred to culture dishes pre-moistened with 5 mL DMEM/F12 (Gibco Invitrogen, USA) containing 15% FBS, 10 ng/ml basic fibroblast growth factor, 10 ng/ml epidermal growth factor, 1× Insulin-Transferrin-Selenium, and 2 mM L-glutamine (All bought from Sigma-Aldrich, USA) [16]. The explants were removed after 10-12 days, and then the cells were subcultured for propagation when reached about 80% confluency.

Immunophenotypic analysis of CL-MSC. Cells were trypsinized, washed, and resuspended in PBS at a concentration of 10^6 cells and stained in the dark at 4^oC for 20 min with FITC or PE-conjugated antibodies as follows: anti-CD13-PE, anti-CD14-FITC, anti-CD19-FITC, anti-CD34-FITC, anti-CD44-PE, anti-CD45-FITC, anti-CD73-PE, anti-CD90-PE, anti-CD105-PE, anti-CD166-PE, and anti-HLA-DR-FITC (All bought from BD Biosciences, USA). Then, the cells were washed two times with PBS to remove excess antibody and resuspended in 500 µl FACSFlow. The fluorescence intensity was evaluated by a flow cytometer (FACSCalibur, BD Biosciences, San Jose, CA, USA). All data were analyzed by using Cell Quest Pro software (BD Biosciences, USA).

Differentiation of CL-MSC into adipocytes and osteoblasts. For differentiation into the adipogenic cells, cells at 70-80% confluence were plated at 1×10^4 cells per well in 6-well plates and were cultured for 2 weeks in previously well-known medium [17]. Adipogenic differentiation was evaluated by observing lipid droplets in cells under a microscope and by staining with Oil Red solution (Sigma-Aldrich, St. Louis, MO, USA). For differentiation into osteogenic cells, cells were also induced in previously published medium for 3 weeks [17]. Osteogenic differentiation was confirmed by RT-PCR for *Osteocalcin* and *Osteopontin* expression. Non-induced cells were served as control groups.

Differentiation of CL-MSC into the endothelial lineage. To induce endothelial differentiation, CL-MSC were seeded at $5 \times 10^4/cm^2$ in fibronectin-coated culture flasks. Following 14 days of induction under these conditions, cells were termed as CL-MSC-derived endothelial-like cells. Differentiation medium consisted of endothelial growth medium 2 (Lonza, USA), 3% FBS, and 50 ng/mL VEGF (Sigma-Aldrich, St. Louis, MO, USA). Both CL-MSC and HUVEC (ATCC, USA**)** were used as negative and positive control groups, respectively [11, 15].

RT-PCR. Total RNA was extracted using Trizol (Sigma-Aldrich, St. Louis, MO, USA). RT-PCR was performed from total RNA using Access Quick™ RT-PCR kit (Promega, USA), under the following

condition: initial reverse transcription at 45°C for 45 min and 95°C for 2 min, followed by 35 cycles of 30 s at 94°C, 30 s at 55-60°C, and 45 s at 72°C. After completing the last cycle, all samples were incubated at 72°C for 10 min. The PCR products were analyzed by electrophoresis with 2% gel agarose, visualized with EtBr staining and photographed by bioimaging system (UVP, USA). Primer sequences are as follow: Osteocalcin F: CTCTGTCTCTCTGACCTCACAG, R: GGAGCTGCTGTGACATCCATAC (360 bp). Osteopontin F: CACCTGTGCCATACCAGTTAAAC, R: ATCCATGTGGTCATGGCTTT (220 bp). VEGFR1 F: GACGTCTAGAGTTTGACACGAAGC, R: GCATGCAAC ACTGAGTAACATGAC (239 bp). VEGFR2 F: CTTACCCCAGGATATGGAG, R: CCG TCAAGGGAAAGACTACG (496 bp). VE-cadherin (vascular endothelial) F: CCCGTCTTTACTCAATCC ACA, R: GGGTTTGATGATACCCTCG TT (496 bp). vWF F: GGCAGCTGTTCTTATGTCCT, R: TGGAA CTCATTGTTTTGTGG (289 bp). CD31 F: GTGAG GGTCAACTGTTCTGT, R: GTGACCAGTTCACTC TTGGT (242 bp). GAPDHhu F*:* ACAGTCAGCCG CATCTTCTT, R: ACGACCAAAT CCGTTGACTC (94 bp).

Immunocytochemical analysis. After endothelial differentiation, cells were washed with PBS, fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.25% Triton X-100 at room temperature for 40 min. They were then blocked with a blocking solution containing 1% BSA and incubated overnight with mouse monoclonal antibody (Ab) against human VEGFR1, VEGFR2, vWF, CD31, and VE-cadherin (All bought from Santa Cruz Biotechnology, USA). The cells were washed with PBS and incubated with PE- or FITC-conjugated anti-mouse IgG (Santa Cruz Biotechnology) for 1 h. In all immunochemistry assays, negative staining controls were carried out by omitting the primary antibody. After washing, cell nuclei were stained with Hoechst 33342 (Sigma-Aldrich, St. Louis, MO, USA) for 10 min. Images were captured using a fluorescent microscope.

In vitro angiogenesis assay. Analysis of capillary formation was performed using the *in vitro* angiogenesis kit (Growth Factor Reduced Matrigel Matrix). Briefly, Matrigel was added to a 96-well plate at a concentration of 50 ml/cm^2 and then incubated at 37°C for 1 hour to allow solidification. After endothelial differentiation, about 5×10^3 cells were suspended in 50 µl of the endothelial growth medium 2 without VEGF, plated onto a 96-well plate pre-coated gel matrix and incubated for 12-24 hours. The formation of capillary structures was observed under a light microscope.

LDL uptake assay. After incubation of cells in endothelial differentiation medium for 14 days, a LDL uptake assay was performed from a previously described report [15]. The differentiated cells were washed with PBS and incubated in a medium containing 10 µg/mL 1,1´-dioctadecyl-1-3,3,3´,3´-tetramethylindocarbocyanine labeled acetylated LDL (Invitrogen, Carlsbad, CA, USA) at 37°C for 24 h. Cells were fixed with 4% formaldehyde and after washing, counterstained with Hoechst 33342 (Sigma-Aldrich, St. Louis, MO, USA). In all functional assays, samples were compared with CL-MSC (negative control) and HUVEC (positive control) under the same protocols.

Karyotype analysis. Before and after endothelial differentiation, cells were subjected to karyotyping analysis. To obtain chromosome preparations, active dividing cells from subconfluent culture flasks were treated with 60 ng/ml colcemid (Sigma-Aldrich) to block or inhibit proliferation of cells. After mitotic arrest, the cells were harvested using 0.25% trypsin/EDTA and immersed in 75 mM KCl at 37°C for 30 min. The cells were collected by centrifugation, the supernatant was replaced with Carnoy's fixative (60% ethanol, 30% chloroform, and 10% glacial acetic acid, Sigma-Aldrich, St. Louis, MO, USA), and the suspension was spread on slides. The samples were stained by Leishman's method. Briefly, slides were stained with freshly made Leishman's stain (0.2% Eosin-Methylene blue in methanol) (Sigma-Aldrich, St. Louis, MO, USA) for 8 min. They were then rinsed in running water for 1 min and air dried. Finally, the slides were cleansed in 2 changes of xylene. The karyotype was analyzed by G-banding techniques and Ikaros software (version 5.0, MetaSystems GmbH, Germany).

RESULTS

Morphology and proliferation ability of CL-MSC. Using a tissue block attachment method [16], scattered, long, and spindle-shaped cells were observed in the gaps between the tissue blocks under an inverted microscope after 7 days (Fig. 1a). After 10-12 days of culture, the cell number in each small colony reached up to a few hundred (Fig. 1b). At that time, the tissue blocks were removed. The cell shape gradually changed to a uniform spindle shape. The cells reached about 80% confluency in culture after approximately 3 weeks. After three passaging, the cells reached homologous fibroblast-like cell population and arranged in parallel arrangement (Fig. 1c). The observed morphologies of CL-MSC were similar to those of bone marrow-derived MSC [17].

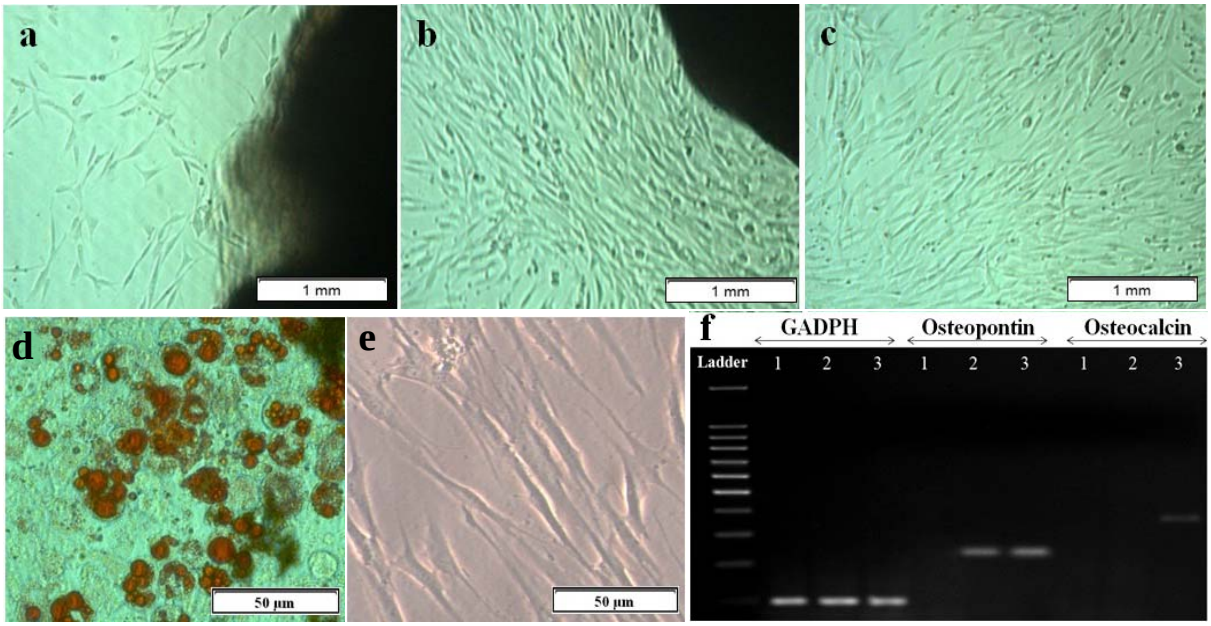

Fig.1. Characterization of CL-MSC. The fibroblast-like cells were observed after 7 **(a)** and 12 days **(b)** of primary culture and the third passage **(c)**. After adipogenic differentiation, cells captured red color with Oil red staining **(d)**, but not in CL-MSC **(e)**. The expression of *Osteocalcin* and *Osteopontin* were detected by RT-PCR. *GAPDH* was used as internal control **(f)**: lane 1, CL-MSC-negative control and lanes 2 and 3, CL-MSC-derived osteoblasts after 1 and 3 weeks, respectively.

Immunophenotypic characteristics of CL-MSC. CL-MSC isolated by the described method [16] were extensively expanded and identified by flow cytometry. The third subcultured cells were negative for CD19, CD34, CD45, HLA-DR markers. In addition, these cells were found to be positive for the markers of CD13, CD44, CD73, CD90 and CD166. Interestingly, they also expressed CD14 (Fig. 2).

Osteogenic and adipogenic differentiation of CL-MSC. In adipogenic medium, MSC began changing into ovoid morphology and accumulating intracellular lipid droplets, which were positive with Oil red staining (Fig. 1d). In control group, cells did not

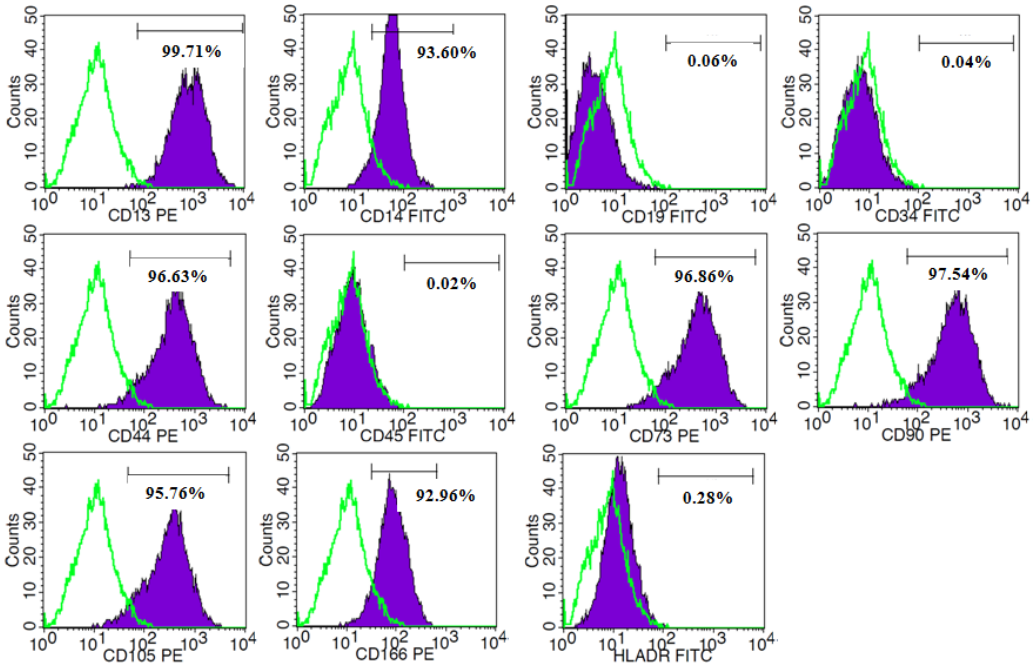

Fig. 2. Detection of MSC-specific marker expression by flow cytometry analysis. CL-MSC were stained with FITC- or PE-conjugated antibodies against the indicated markers. All experiments were repeated in triplicate.

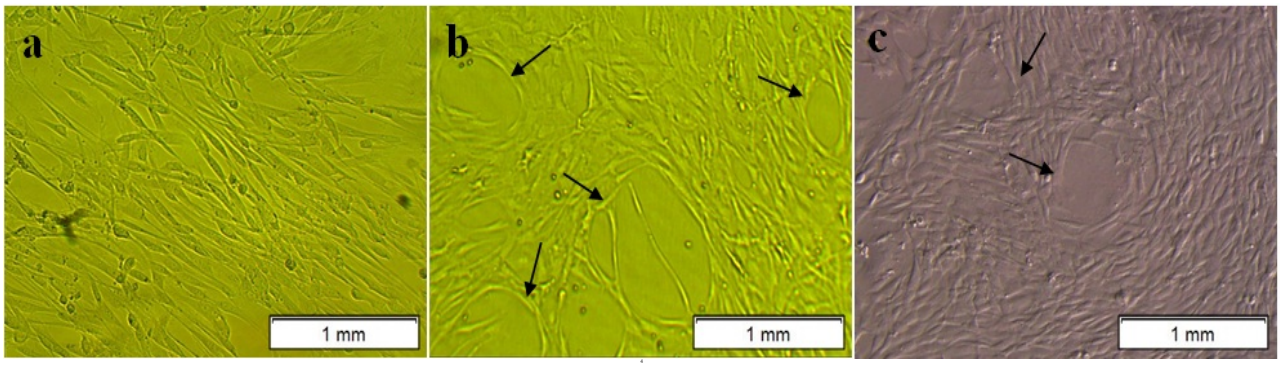

Fig. 3. Morphological changes of CL-MSC in endothelial differentiation. Two weeks after endothelial differentiation, cells became slightly shorter and broader those of CL-MSC **(a)**. Some lumen-like structures were spontaneously formed by differentiated cells derived from CL-MSC (**b** and **c**).

differentiate (Fig. 1e). When induced to differentiate under serum-free osteogenic conditions, the spindle shape of these cells flattened and broadened with increasing time of induction. The expression of *osteopontin* was detected at week 1 and week 3, whereas *osteocalcin* only was expressed in the osteoblast phenotype of differentiated cells at week 3. The undifferentiated cells did not exhibit these genes (Fig. 1f).

Morphological change analysis of CL-MSC-derived endothelial-like cells. After two weeks of differentiation, cells remained attached to the culture flasks, and retained their parallel or whirlpool arrangements. The morphological changes were not apparent upon differentiation, although the volume of the CL-MSC-derived endothelial-like cells was slightly shorter and broader than that of CL-MSC. Some of the differentiated cells adopted an irregular arrangement and tended to form lumen-like structures spontaneously *in vitro* (Fig. 3b and Fig. 3c), while the undifferentiated cells did not exhibit (Fig. 3a).

Detection of endothelial-related gene expression by RT-PCR. The mRNA expression of endothelial-specific genes, including *CD31*, *vWF*, *VE-cadherin*, *VEGFR1*, and *VEGFR2* was detected in differentiated cells by RT-PCR (Fig. 4, lane 2). The results were the same as HUVEC gene expression (lane 3). CL-MSC did not express these genes (lane 1).

Detection of endothelial-specific marker expression by immunocytochemistry. Cells were examined by immunocytochemistry for expression of the five endothelial-specific markers. Differentiated cells showed a fluorescent signal of the five markers: VEGFR1, VEGFR2, vWF, CD31, and VE-cadherin (Fig. 5b, d, f, h, and j). CL-MSC did not show any positive signal after they were cultured in the growth medium as negative controls (Fig. 5a, c, e, g, and i). These immunocytochemical data confirmed the RT-PCR results.

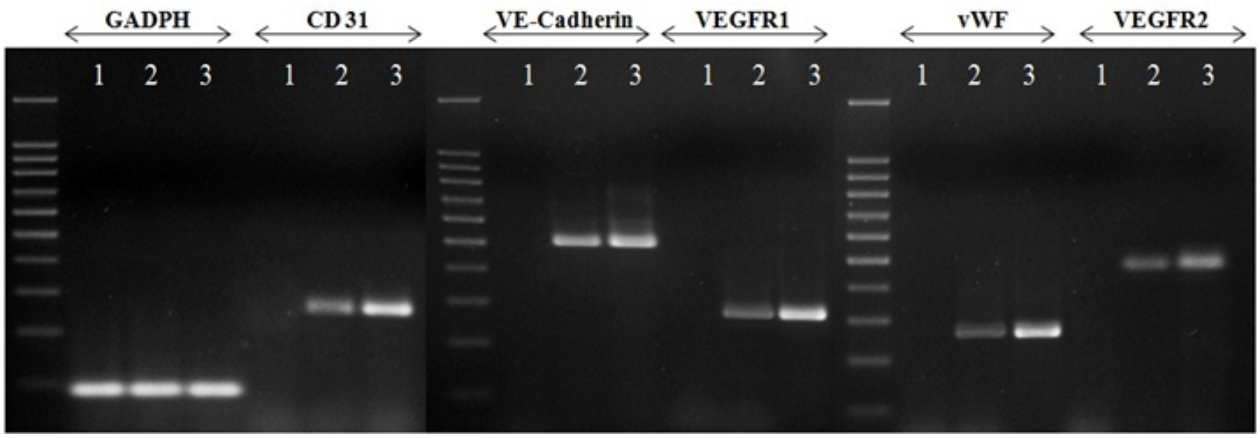

Fig. 4. Detection of endothelial-specific gene expression by RT-PCR. The expression of five endothelial-specific genes (*CD31*, *VE-cadherin*, *vWF, VEGF1*, and *VEGFR2*) were detected in both CL-MSC-derived endothelial-like cells (lane 2) and HUVEC (lane 3) by RT-PCR, while CL-MSC did not exhibit them (lane 1). *GAPDH* was used as an internal control.

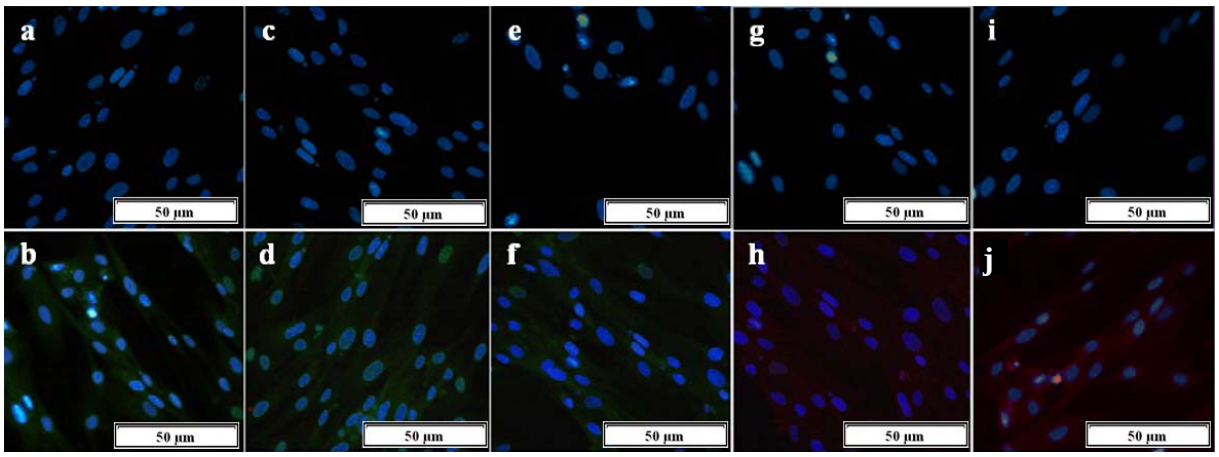

Fig. 5. Detection of endothelial-specific marker expression by immunocytochemical analysis. CL-MSC (**a**, **c**, **e**, **g**, and **i**) and CL-MSC-derived endothelial-like cells (**b**, **d**, **f**, **h**, and **k**) were stained with mouse monoclonal antibodies against VEGFR1 (a and b), VEGFR2 (c and d), vWF (e and f), CD31 (g and h) and VE-cadherin (i and j). Cells were stained with PE- or FITC-conjugated anti-mouse IgG. Cell nuclei were stained with Hoechst 33342.

Detection of capillary formation by Matrigel angiogenesis assay. Differentiated cells cultured without VEGF showed the capillary network formation (Fig. 6a) that is similar to HUVEC in the same protocols (Fig 6b). On the contrary, the undifferentiated CL-MSC showed very few capillaries and most of the cells stayed flattened in the medium (Fig 6c).

Detection of LDL-uptaking by LDL-uptaking assay. Both differentiated cells (Fig. 7a) and HUVEC (Fig. 7c) could uptake DiI-Ac-LDL into the cytoplasm after cells were incubated in the medium containing DiI-Ac-LDL. However, CL-MSC were unable to uptake DiI-Ac-LDL after they were cultured in the growth medium as negative controls (Fig. 7b).

Analysis of cell karyotype. Chromosome samples were prepared and analyzed using G-banding technique and chromosome automatic analysis system (Ikaros, Version 5.0 software, MetaSystems GmbH, Germany). CL-MSC and CL-MSC-derived endothelial-like cells showed normal diploid karyotype, i.e. 46 XY. Chromosome structural abnormalities such as deletion, inversion, translocation, and ring chromosome, were not observed by karyotypic analysis of G-banding (Fig. 8).

DISCUSSION

There is growing need for novel technologies to restore, maintain, and enhance organ function, and stem cells, especially MSC, have emerged as a new venue for regenerative medicine. While hematopoietic stem cells can be further enriched by immunomagnetic isolation based on specific surface antigens like CD34 or CD133, MSC lack a unique surface antigen that can be used for positive selection. Therefore, the general strategy for the enrichment of MSC has been based on the adherence of cells to plastic culture dishes [6].

They are also mainly characterized by their ability to

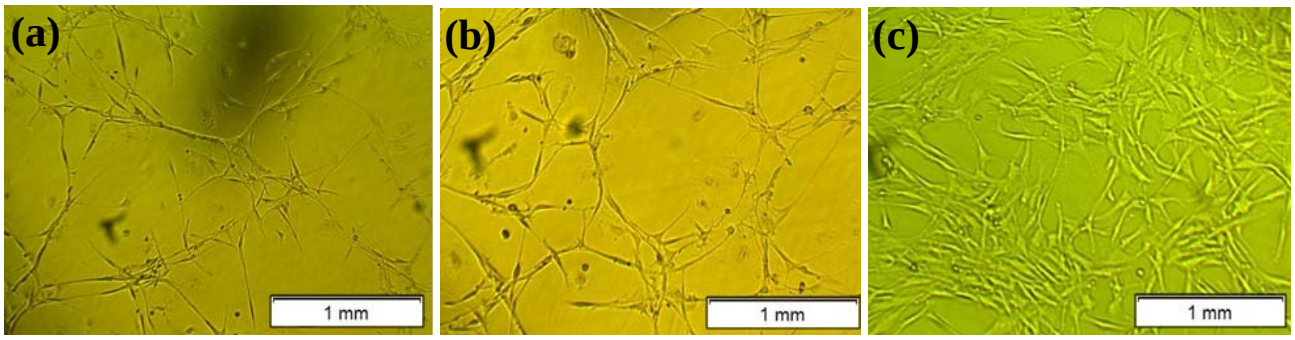

Fig. 6. *In vitro* angiogenesis assay. The capillary networks were formed in endothelial growth medium 2-medium by CL-MSC-derived endothelial-like cells **(a)** and HUVEC (positive control) **(b)**, but not in CL-MSC (negative control) **(c)**.

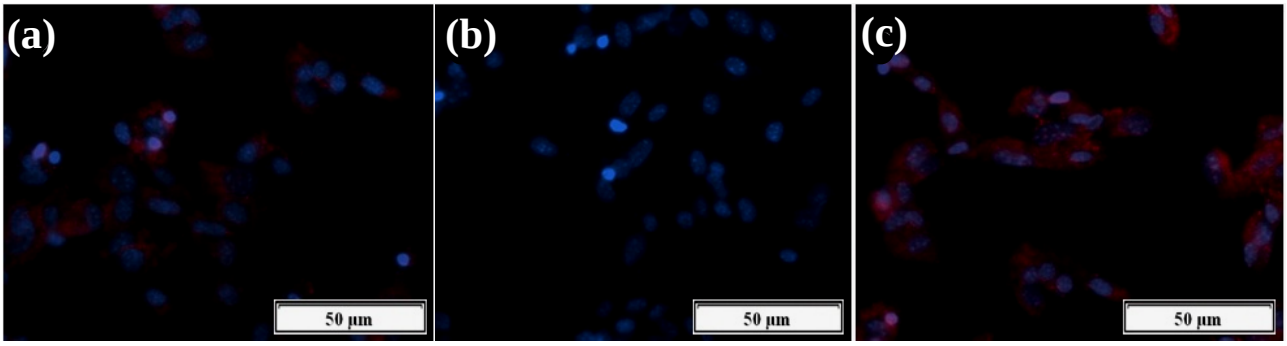

Fig. 7. LDL-uptaking assay. Differentiated cells were incubated in the differentiation medium containing DiI-Ac-LDL for 24 hours. After nuclei were stained with Hoechst 33342, cellular incorporation of DiI-Ac-LDL was detected by fluorescence microscopy **(a)**. The undifferentiated CL-MSC **(b)** and HUVEC **(c)** were also incubated with DiI-Ac-LDL for 24 hours as negative and positive controls, respectively.

differentiate into osteocytes, chondrocytes, and adipocytes under *in vitro* controlled conditions. On the other hand, MSC fulfill all criteria of true stem cells, including self-renewal, multilineage differentiation, and immunophenotypic character [18].

MSC population might be easily contaminated with fibroblasts and mature mesenchymal cells, if isolated from connective tissue. These cells were the most frequent contaminating cell phenotype present in many cell culture systems [19]. Therefore, analysis of profile marker expression, both negative and positive markers, is essential. The present study showed isolated CL-MSC were negative for CD19 (B lymphocyte antigen), CD34 (hematopoietic stem cell antigen), CD45 (leukocyte common antigen), and HLA-DR, indicating that there is no hematopoietic stem cell lineage in the analyzed cell populations. Besides, CL-MSC expressed a set of markers on their surface, including CD13, CD44, CD73 (SH3, SH4), CD90 (Thy-1), CD105 (SH2 or endoglin), and CD166 (Fig. 2). These markers are considered (important markers to identify MSC [17, 18]. Surprisingly, we also observed, in this study, that CD14 expression on CL-MSC is different from immunophenotypic characteristics for defining human MSC [18]. However, this result was consistent with the Kita *et al.* study [16]. It is possible that the cells might acquire CD14 expression after the isolation from a tissue, and expression CD14 might be a unique property to distinguish CL-MSC from other adult tissues-derived MSC. In addition, CD14 might function as a factor for MSC to explain how MSC can home to injured or infected sites [16]. Stem cell therapy for treatment of vascular-related diseases is one of the most important research areas in

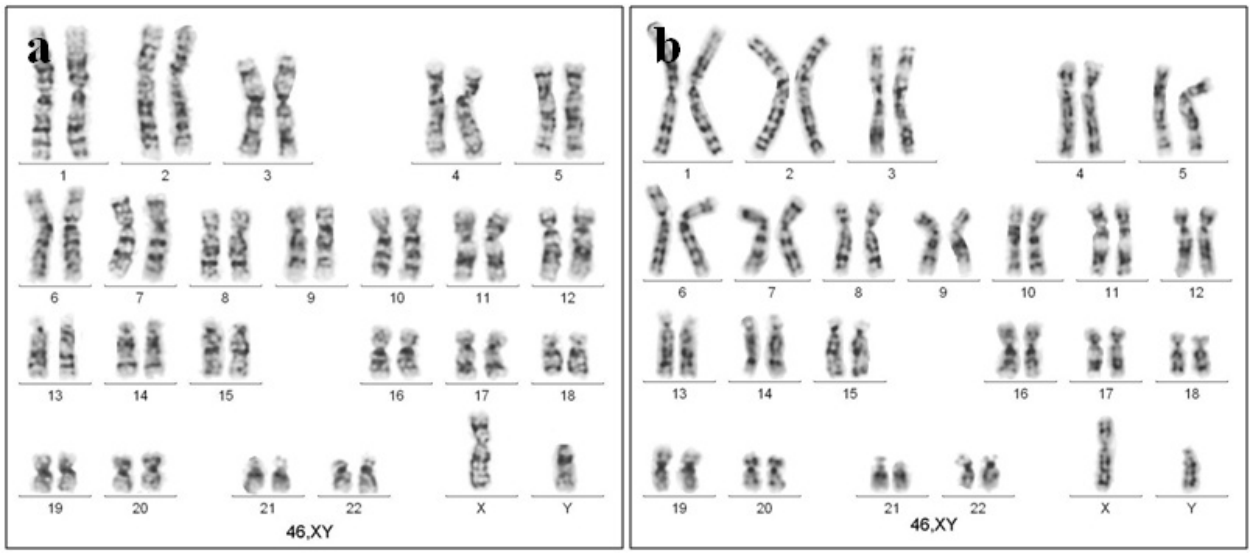

Fig. 8. Representative karyotypes of CL-MSC and endothelial-like cells from CL-MSC. The results of the standard G-banded karyotype analyses showed that no chromosomal aberrations were observed in both CL-MSC **(a)** and endothelial-like cells from CL-MSC **(b)**. Twenty two pairs of somatic chromosomes as well as one X chromosome and one Y chromosome (totally 46 chromosomes) have been shown in the Figure.

tissue engineering. The use of MSC-derived endothelial cells seems attractive for the development of engineered vessels and may also be useful to augment vessel growth in ischemic tissue [9]. In this study, we could also obtain endothelial cells by differentiating CL-MSC in a medium supplemented with growth factors. In this protocol, we separated and discarded the umbilical cord vein and umbilical cord arteries before culture; therefore, contamination of endothelial cells from umbilical cord lining or vessel wall might be avoided. With suitable condition based on previously published reports [9, 10, 15], we demonstrated that CL-MSC might differentiate into endothelial-like cells *in vitro*. Interestingly, endothelial differentiation of CL-MSC appeared to proceed without a noticeable morpho-logical change after two weeks of differentiation.

To assess endothelial differentiation, we chose to determine the expression of some endothelial-related markers that play important roles in physiologic processes of endothelial differentiation or endothelium maturation. CL-MSC showed expression of CD31, vWF, VE-cadherin, VEGFR1, and VEGFR2 in both mRNA and protein levels after a two-week induction. The results were the same as gene expression of HUVEC. A number of studies have proved that the four endothelial specific molecules (vWF, VE-cadherin, VEGFR1, and VEGFR2) play an important role in angiogenesis process, especially endothelium maturation [9-11, 15]. VEGFR1 and VEGFR2 are two major VEGF receptors. However, the precise molecular mechanisms of how VEGFR works with VEGF, an important growth factor for the endothelial differentiation, have not been completely understood yet [20]. Differentiation of MSC in the presence of VEGF also up-regulates the expression of the VEGF receptors, which not only play a major role in angiogenesis *in vivo* but also associate with matrix-metalloproteases in forming capillary-like structures *in vitro* [21]. In this study, we also identified an enhanced ability to form capillary-like structures *in vitro* of CL-MSC in Matrigel after endothelial differentiation. In addition, VEGF is also essential for vascular progenitor cell differentiation in early embryogenesis and vasculogenesis [20]. Therefore, VEGF is often used as the primary stimulus for chemical-mediated differentiation of stem cells into endothelial cells *in vitro*, which might be a useful model to explain the role of VEGF in differentiation and maturation process of endothelial cells.

vWF is synthesized by vascular endothelial cells and considered as one of the endothelial-specific markers. vWF is expressed in normal endothelial cells while its expression is reduced significantly in damaged vascular endothelial cells. Therefore, this implied that vWF could be a key factor for repair of damaged ischemic vascular diseases [22]. VE-cadherin or CD144 was proved to be necessary for vascular genesis and repair of damaged vascular diseases [23]. The expression of these four markers in differentiated CL-MSC seems to contribute to the promotion of endothelial differentiation as compared with differentiated bone marrow MSC [10] and amniotic fluid stem cells [24]. We also observed that the expression of CD31(also known as PECAM-1) and ability of LDL uptake in differentiated cells. According to the research of Oswald *et al.* [10], CD31 expression was not observed after induction. However, in other studies, the CD31 expression level had apparently increased after endothelial differentiation [13, 24]. CD31 is also considered a later endothelial differentiation marker and the up-regulation of CD31 may be enhanced by elongation of the differentiation time course [10]. This discrepancy may be explained by differences in culture conditions that can lead partial differentiation of endothelial cells with distinct phenotype. Uptake of LDL by endothelial cells largely occurs by endocytosis. The advantages of using LDL metabolism as a marker are allowing labeling of live endothelial cells without fixation or permeabilization for optimum staining that does not affect cell viability. Therefore, the use of fluorescently labeled LDL will allow for isolation or identification of endothelial cells from the different cell population [24].

In this study, we also evaluated karyotype of cells because the long-term culture and differentiation process could modify the cell cycle and chromosome stability, Therefore, the alterations of the chromosome structure and DNA replication or repair mechanisms might be happened. Practically, karyotype is a reliable indicator for evaluating genetic stability or transformation of cultured cells [25]. Kunkel and Bebenek [26] proved that there is one chance of gene mutation at every single cell division, as Ueyama's recent study verified human MSC [25]. In addition, there have been concerns about tumorigenesis of stem cells or mature cells-derived stem cells after they are highly expanded *in vitro* or transplanted *in vivo*. Therefore, it is important to examine the cytogenetic stability of cells before clinical application.

In conclusion, our findings suggested that CL-MSC could be isolated by a simple tissue culture technique. They also could differentiate into endothelial-like cells *in vitro*, which expressed some endothelial-specific markers and displayed enhanced capacity to form capillary-like structures and uptake LDL without significant changes in their karyotype. Consequently, CL-MSC are suitable for basic and clinical studies to cure several vascular-related diseases. We will further investigate the endothelial differentiation mechanism of CL-MSC in our future research.

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