3D Study of Capillary Network Derived from Human Cord Blood Mesenchymal Stem Cells and Differentiated into Endothelial Cell with VEGFR2 Protein Expression

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Abstract

Background: New blood forming vessels are produced by differentiation of mesodermal precursor cells to angioblasts that become endothelial cells (ECs) which in turn give rise to primitive capillary network. Human cord blood (HCB) contains large subsets of mononuclear cells (MNCs) that can be differentiated into endothelial-like cells *in vitro*.

Materials and Methods: Human mononuclear progenitor cells were purified from fresh umbilical cord blood by the expression of CD34 and FLK-1 antigens expressed in both angioblasts and hematopoetic stem cells. The HCB derived mesenchymal stem cells (MSCs) can differentiate into adipocyte, osteocyte, chondrocyte and ECs.

Results: In this study, the differentiation of human cord blood mesenchymal stem cells (hCBMSCs) into endothelial-like cells was induced in presence of vascular endothelial growth factor (VEGF) and insulin-like growth factor (IGF-1). The differentiated ECs were then examined for their ability to express VEGF receptor-2 (VEGFR2) and von Willebrand factor (vWF). These cells were adopted to grow, proliferate and develop into a capillary network in a semisolid gel matrix *in vitro*.

Conclusion: The capillary network formation in each well of 24-well plate was found to be 80% in presence of VEGF (40 ng/ml) and IGF-1 (20 ng/ml) of culture media, suggesting that the capillary network formation is associated with endothelial-like cells derived from hCBMSCs by expression of their markers.

Keywords: Human umbilical cord blood (HUCB); Mesenchaymal stem cell (MSC); Angiogenesis; Differentiation; IGF-1

Introduction

Endothelial progenitor cells from cord blood play an important role in various physiological and pathophysiological processes (1). They participate in angiogenesis and vascular repair (2, 3-4), angiogenesis, wound healing, reproductive tissue cycles, and tumorogenesis (4). Promising therapeutic strategies are based on the concept of endothelial stem cells being proliferated into mature endothelial cells (ECs). In this study, cord blood derived mono-nuclear cells

(MNCs) and mesenchymal stem cells (MSCs) have been demonstrated to trans differentiate into mature ECs following the construction into capillary network (5-7). The functionality of the cells was confirmed by their ability to form capillary network in presence of vascular endothelial growth factor (VEGF) (8). Differentiation of the ECs from different sources inducing the angioblast in the embryo (8), the endothelial progenitor cells (EPCs), mesoangioblasts

and multipotent progenitor cells- (MAPCs) or side population in the adult bone marrow (2, 9) and murine embryonic stem cell line (10) has been reported (11). Various surface markers such as CD34, CD146, vascular endothelial growth factor 2 (VEG FR2) which is sometimes referred as KDR, tie 2, endothelial cadherin and E-selection have been used to identify the stem cell contributing to angiogenesis (12-14).

Nonetheless function of the cells was confirmed by showing their ability to form capillary network in presence of VEGF (7). In this study, we have explored the differentiation process of hCBMSCs to ECs by adding insulin-like growth factor-1 (IGF-1) in addition to VEGF as well as the effects of IGF-1 on the differentiated ECs migration by evaluation of CXCR4 protein expression. The expression of VEGF receptor-2 (VEGFR2), CD31 and von Willebrand factor (vWF) was determined at protein level using immunocytochemical (ICC) and reverse transcription polymerase chain reaction (RT-PCR) methods, followed by development of capillary network formation, confirming the rate of functionality of the cells (14-15).

Materials and Methods
Reagents and antibodies
Medium supplemented with Trace Elements 131 (MCDB 131), ECM gel solution, phycoerythrin (PE) conjugated anti-mouse IgG and 4´, and 6-diamidino-2-phenylindole (DAPI) were purchased from Sigma (St. Louis, USA). Anti-CD44, anti-CD73, anti-CD105, anti-CD34, anti-CD31, anti-vWF were purchased from eBioscience (USA) while VEGF and IGF-1 were from Peprotech (NJ, USA). Ficoll-Hypac, penicillin, streptomycin and Trypsin-EDTA were also purchased from Biochrom (Berlin, Germany). Dullbecco's Moified Eagle's Medium-High Glucose (DMEM-HGL), GlutaMAX-ITM, fetal bovine serum (FBS), bovin serum albumin (BSA) and α-Minimal Essential Medium (α-MEM) were from the Gibco Invitrogen (Carlsbad, CA, USA). Oil red –O– staining, alkaline phosphatase staining (ALP) kits were purchased from Sigma (St. Louis, USA).

Human umbilical cord blood (HUCB) collection
No complications were observed in human umbilical cord blood (HUCB) sample collection, and each sample (about 45–55 ml) was collected from fresh placentas with attached umbilical cords by gravity flow. Heparin was used as anticoagulants for storage with transport temperature of 15–22°C, within 2-4 h period. MSC, with morphology of adherent fibro-blastoid spindle shaped cells culture was based on previously reported methods (16-18). The method was modified as follows: The Ficoll-Hypac (d=

1.077g/ml) was layered over a cord blood collection (10-12 ml) and centrifuged at 1900 rpm for 30 min at room temperature. MNCs at the interface (gray loop) were recovered and washed twice in phosphate buffered saline (PBS). These cells were plated at 25 cm^2 culture flasks containing DMEM–HG, supplemented with 10% FBS, 2 mM GlutaMAX-ITM (L-analyl-L-glutamine), 10 U/ml penicillin and 100 µg/ml stereptomycin. The cultures were then placed in an incubator at 37 °C and 5% CO_2 in air. After 48 h, non- adherent cells were removed by washing with PBS. The proliferation medium was changed 2 times per week subsequently. After 3 weeks, the culture reached 80% confluency. The cells were harvested by per cm^2 of surface area.

Isolation and propagation of hCBMSCs
Cord blood samples colonies with the classical MSC and with morphology of adherent fibroblastoid spindle shaped cells culture were based on previously reported methods (16-19). The method was modified as follows: The Ficoll – Hypac (d=1.077g/ml) was layered over a cord blood collection (10-12 ml) and centrifuged at 1900 rpm for 30 min at room temperature. MNCs at the interface (gray loop) were recovered and washed twice in phosphate buffered saline (PBS). These cells were plated at 25 cm^2 culture flasks containing DMEM – HG, supplemented with 10% FBS, 2 mM GlutaMAX-ITM (L-analyl-L-glutamine), 10U/ml penicillin and 100 µg/ml stereptomycin. The cultures were then placed in an incubator at 37 °C and 5% CO_2 in air. After 48 h, non-adherent cells were removed by washing with PBS. The proliferation medium was changed 2 times per week subsequently. After 3 weeks, the culture reached 80% confluency. The cells were harvested by per cm^2 of surface area.

Transdifferentiation of HCBMSCs to osteogenic and adipogenic
In the present study, we carried out trans-differentiation of MSCs to osteocytes and adipocytes in order to confirm the source and process of differentiation. The proliferation medium was replaced with an osteogenic medium induced by hCBMSCs for two weeks under the influence of DMEM composed of 10% FBS, 50µg/ml ascorbic Acid 2-phosphate (5, 19), 0.1 µM dexamethasone (Sigma Chemical Co.) and 10µM β–glycerol- phosphate (Sigma Chemical Co.) (20). The culture was then placed in an incubator at 37 $^\circ$C and 5% CO_2 for 2 weeks, with medium changes two times per week. Control cultures without the differentiation inducer were maintained in parallel to the differentiation experiences. Both differentiated and undifferentiated cells were stained with alkaline phophatase (ALP)

using a commercial Kit. For adipogenesis, the proliferation medium was replaced with a differentiation medium consisting of DMEM, supplemented with 10% FBS, 1 µM dexamethasone (Sigma Chemical Co.) (21), 200 µM indomethacine (Sigma Chemical Co.), 1.7 µM insulin, 500 µM isobutyl-methylxanthine, 0.05 U/ml penicillin and 0.05 µg/ml streptomycin for 2 weeks. The culture was then placed in an incubator at 37 $^\circ$C and 5% CO_2 for 2 weeks, with medium changes 2 times per week. For adipocyte identification, intracellular accumulation of these cells was stained with oil red-o-staining. Briefly, the cells were fixed in 10% solution of formaldehyde in aqueous phosphate buffer for 1 h, washed with 60% isopropanol and stained with oil red-o solution for 10 m (22-23).
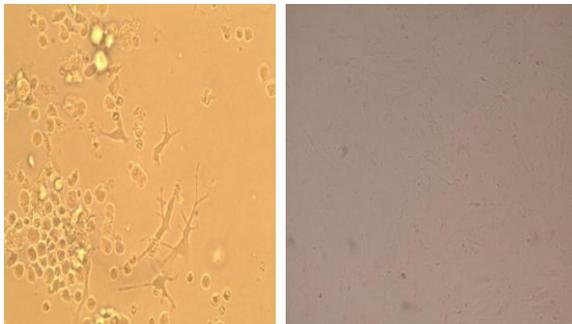

Figure 1. Characterization of human umbilical cord blood-derived mesenchymal stem cell. Homogeneous cell population with fibroblast-like or spindle-shape morphology obtained on day 7.

Immunophenotyping of hCBMSCs and the differentiated cells

Proliferated hCBMSCs and differentiated ECs were detached from the culture flasks using PBS (pH 7.4) containing trypsin (0.05%) and EDTA (0.02%), and washed once with DMEM and once with PBS. The cell viability was determined with trypan blue staining, and dead cells were counted with a Neubauer slide. The cells (3×10^6/ml) were resuspended in 50 ml PBS and incubated with either fluorscein isothiocyanate (FITC) or PE-conjugated antibodies in dark for 45 min at 4 $^\circ$C. The following antibodies were used for immune- phenotyping: anti-CD44-PE, anti-CD73-PE, anti-CD105-FITC, anti-CD34-FITC, anti-FLT1-FITC, anti-VE-cadherin-FITC, anti-Tie2-FITC, anti-vWF-FITC, and anti-CD31-FITC. At the end of the incubation period, cells were washed twice with PBS containing 2% BSA and fixed with 1% paraformaldehyde solution in PBS. Mouse isotype antibodies served as negative control. The analysis was performed through a flow cytometer (Partech, Germany).

Induction of the hCBMSCs differentiation to endothelial-like cells

hCBMSCs were conducted by cultivating in the MCDB131 media containing 5% FBS, 20 ng/ml IGF-1, 40 ng/ml VEGF, and incubated at 37 $^\circ$C for 7 days. Cells were fed every 3 days. After induction of differentiation, the cells were allowed to develop capillary-like network formation on ECM gel solution. Briefly, 200 µl ECM gel solution was added to each well of a 24-well microplate and incubated for 4 h at 37 $^\circ$C. The induced hCBMSCs (6×105) were plated onto ECM gel and incubated for 7 days. Thereafter, on day 14 the area of tubulogenesis was assessed by counting the number of tubes in each well under phase contrast microscope (Nikon, Japan).

Immunocytochemical analysis (ICC)

The differentiated ECs were fixed with 4% paraformaldehyde in PBS containing 0.1% Triton X-100 for 15 min at room temperature. Possible non-specific bindings were removed by several washings with PBS containing 1% BSA. The cells were then incubated overnight at 4 $^\circ$C separately with primary antibodies produced against vWF (1: 100) and VEGFR2 (1: 500). The cells were then washed three times with PBS and incubated with PE-conjugated anti-mouse IgG (1: 100) for 1 h at 37 $^\circ$C. Finally, the cells were counterstained with DAPI (1: 1000) prior to observation under a fluorescence microscope (Nikon, TE 2000, Japan).

Reverse transcriptase–polymerase chain reaction (RT-PCR)

Total RNA was extracted from the differentiated ECs using guanidine thiocyanate. The samples were subjected to reverse transcriptase (RT) reaction to synthesize the first cDNA strand. cDNA was then amplified by Taq DNA polymerase dissolved in PCR buffer (Qiagen, USA) in a 50 µl reaction mixture containing 0.2 mM/L dNTPs and 40 pM of VEGFR2 primers: sense; 5´- CTGGCATGGTCTTCTGTG-AAG -3´, antisense; 5´- AATACCAGTGGATGTGA-TGC -3´, and vWF primers: sense: 5´- AATGTT-GTGGGAGATGTTTGC-3´, antisense: 3´- GTG-GATATATCCACCTCTACTTCAGAC -3´.

PCR profile consisted of 5 m of initial denaturation at 94 $^\circ$C, followed by 25 cycles of 30 s of denaturation at 94 $^\circ$C, 60 s of annealing at 55 $^\circ$C, 45 s of extension at 72 $^\circ$C and a final extension step of 10 m. An aliquot of the PCR product (20 µl) was separated by electro- phoresis on a 2% agarose gel and stained with ethidium bromide. β-actin, a housekeeping gene was amplified in the same samples and considered as internal control. Human umbilical vein endothelial cell line (HUVEC) was processed for mRNA

extraction and RT-PCR and considered as positive control.

Results

Characterization of hCBMSCs by flow cytometry
The hCBMSCs separation was performed based on standard methods for the isolation of mononuclear cells, and phase contrast microscopy results in passage P_0 monitored the fibroblast-like spindle shape morphology. To generate passages ($>P_5$), the spindle-shaped cells were analyzed by flow cytometry for presence or absence of hematopoietic and endothelial markers. The hCBMSCs and cell lines known to be positive or negative for the surface markers were used as controls. The hCBMSCs typically expressed the surface antigens CD105, CD44 and CD73 but cells negative for CD34, specific marker of ECs such as VE-Cadherin and VEGF receptors (FLT1, VEGFR2), were not detected in the hCBMSCs.
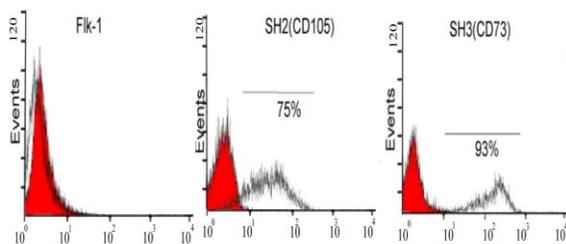

Figure 2. Characterization of HUCB using flowcytometry. Histograms show the immunophenotypes of HUCB isolated from human cord blood.

Differentiation of hCBMSCs into osteoblasts and adipocytes

HCBMSCs under osteogenic inductive medium led to the appearance of refractive crystals in the cells after 2 weeks. The hCBMSCs cultures in adipogenic differentiation medium after 2 weeks led to appearance of round cells presenting numerous fat vacuoles in cytoplasm. These lipid droplets were oil red-o-positive, while untreated control cultures did not show such lipid droplets. By the end of the second week of stimulation, most of the hCBMSCs also became ALP positive. Untreated control culture grown in regular medium without any osteogenic differentiation stimuli did not have spontaneous osteoblast formation even after 3 weeks of culture.

Characterization of the differentiated ECs
In the present study, the effect of growth factors (VEGF and IGF-1) was examined on the differentiation process as well as the changes in the endothelial markers. The VEGF alone with increased expression of the specific markers, immunocytochemistry, flow

cytometry and RT-PCR were the techniques used to confirm the differentiated ECs. Flow cytometry data obtained from ECs derived from hCBMSCs showed that they typically express CD31, vWF, VEGFR2, FLT1, Tie2, VCAM1 and VE-Cadherin as the specific endothelial markers. The amplified products of VEGFR2 and vWF genes were visualized as two bands of 790 and 322 bp, respectively.
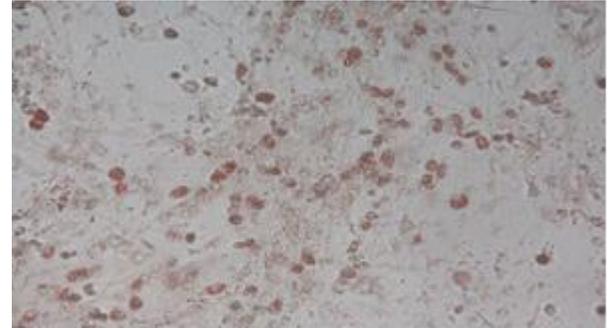

Figure 3. Capillary-like network formation was measured on day 14 of differentiation (magnification 10X).

The band of 193 bp showing β-actin-specific mRNA (internal control) appeared in differentiated ECs as well as hCBMSCs. Expression of endothelial specific genes (VEGFR2 and vWF) was observed in samples obtained from the differentiated cells, while hCBMSCs failed to express VEGFR2 and vWF. The ICC assays revealed that unlike the differentiated hCBMSCs, most of the endothelial-like cells (~80%) were positive for VEGFR2 and vWF. RT-PCR products also showed that, unlike hCBMSCs, the differentiated ECs fairly expressed the VEGFR2 and vWF.

Table 1. Comparison of the capillary network formation potential of the cells before and after differentiation

hCBMSC	Growth factor	Capillary network Formation (%)
Undifferentiated	-	30±1
Differentiated	VEGF	75±1
Differentiated	VEGF+IGF-1	80±5

The MSCs can be an alternative source for EC differentiation. The relative ease of isolating MSCs from UCB and the great plasticity of the cells make them ideal tools for an autologous or allogenic cell therapy. The obtained result indicated that the structures and foundations of ECs, differentiated from human UCB stem cells, were limited to the surface antigen markers and the endothelial specific proteins particularly vWF and VEGFR2 (7).

Nonetheless the present study explored the endothelial-like cells derived from human UCB stem cells for their ability to form *in vitro* capillary network. This fact was more evident with markers specifically expressed with the development of capillary structures. Manipulation of the culture media used for the differentiation was used as a tool to improve the performance of endothelial-like cells. Addition of VEGF in combination with IGF-1 proved to be efficient in promotion of ECs.
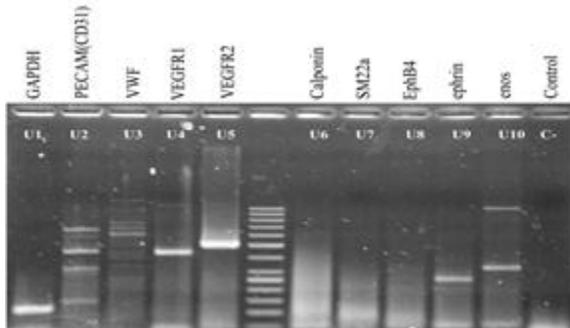

Figure 4. Expression of endothelial specific genes using RT-PCR. (A) RT-PCR results of specific endothelial genes in HUVEC as positive control and the differentiated hCBMSC. (B) VEGFR2 and vWF specific mRNA is not expressed in undifferentiated hBMSC. The product size for VEGFR2 and vWF were 790 and 322 bp, respectively.

The use of VEGF together with IGF-1 probably enhances the viability of cells. Our experience showed that the differentiation in presence of IGF-1 increases the viability of cells and the cells remain functional utile day 14-15 of differentiation. However, the cells differentiated in absences of IGF-1 undergo ng/ml, respectively. Percentage of capillary network formation is defined as the number of cells capable of forming tubular structures.

Discussion

It has been shown that HUCB contains a significant number of primitive hematopoietic precursor cells (HSC) which may form poorly characterized UCB. We studied proliferation and endothelial differentiation of UCB granulation more readily on day 8-10 of differentiation. These data are in accordance with the results reported by Tögel et al. (24).

Various parameters were undertaken to identify the endothelial-like cells from their progenition hCBMSCs. Expressions of CD31, vWF, Tie 2, VE-cadherin and VEGF receptors (VEGFR2 and FLT1) in hCBMSCs and the differentiated ECs confirm these data. The ability of cells to express endothelial specific markers particularly VEGFR2 and vWF is common to all the ECs, which provides evidences to show the ability of cells to develop vascular system. Development of vesicular system which begins on day 8 of

differentiation was associated with the formation of intracellular lumens. Our data presented in this study suggest that the characterization of ECs differentiated from hCBMSCs is associated with features of cells and inducing factors capable of forming capillary network.

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