



Comparative Preliminary Analysis of Umbilical Cord Blood-, Healthy Adult Peripheral Blood-, and Myocardial Infarct Peripheral Blood-derived Endothelial Progenitor Cells

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Abstract

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BACKGROUND: Cardiovascular disease is a leading cause of death globally with the 287,000 deaths per years, characterized by declining of heart function caused by the reduction of heart capacity and lead to heart failure. Cell therapy using endothelial progenitor cells (EPCs) has a big potential for cardiovascular regeneration. EPCs are cells that have ability to differentiate into endothelial cells that can be mobilized and integrated into the defected blood vessel by angiogenesis.

AIM: We aimed to seek the superior EPCs derived from MNCs for functional improvement of advanced heart failure patient by cell therapy using EPCs.

MATERIALS AND METHODS: We did preliminary analysis to compare umbilical cord blood (UCB), healthy adult peripheral blood (PB)-, and myocardial infarct PB-derived EPCs characteristic and surface phenotypes. Different sources of each EPCs mononuclear cells were characterized by the expression of endothelial (cluster of differentiation [CD] 31, acetylated low-density lipoprotein, and von Willebrand) and hematopoietic stem cell (CD45, CD34, and CD133) surface markers with flow cytometry.

RESULTS: In this study, EPCs and the conditioned medium (CM) have been produced and characterized in laboratory scale by comparing several sources of EPCs for instance UCB, PB from healthy people, and patients with myocardial infarction. We have shown that EPC characterizations from each group were not significantly different, but vascular endothelial growth factor and hepatocyte growth factor in UBC-derived CM-EPCs were higher than in PB.

CONCLUSION: In conclusion, the UBC-derived EPCs might have a better potential for cardiovascular regeneration.

Introduction

Myocardial infarction (MI) results in reduction of heart pumping capacity and subsequently lead to heart failure. Despite the innovation in heart failure therapies including drugs and devices, but the morbidity and mortality of heart failure remain high. Patients with heart failure patients were responsible for 11 million physician visits each year and more hospitalizations than all forms of cancer combined [1]. More than half of myocardial infarction develop heart failure and die within five years. Heart failure contributes approximately 287,000 deaths a year. Sudden death is common in heart failure patient (6-9x vs general population). Heart failure contributes approximately 287,000 deaths a year. Sudden death is common in heart failure patient (6-9x vs. general population). Moreover, there was 12%

decreased of deaths from heart failure per decade over the past 50 years [2], [3], [4], [5]. Due to that, myocardial regeneration by increasing number of myocardial cell has potentially to improve heart failure prognosis.

Circulating progenitor cells (CPCs) are mononuclear cells (MNCs)-derived primarily from the bone marrow that contributes to vascular repair and regeneration largely through paracrine mechanisms [6]. CPCs can be measured as MNCs expressing the cluster of differentiation (CD) 34 epitope; these cells have the potential to differentiate into endothelial, hematopoietic, and non-hematopoietic phenotypes [7]. Recently, one study demonstrated that low CPCs count as an indicative of impaired endogenous regenerative capacity [8]. It is generally accepted that circulating endothelial progenitor cells (EPCs) exist in the adult heart, and that they might be involved in restoration of cardiac vasculature after injury [9]. Therefore, this

was a preliminary analysis to compare umbilical cord blood (UCB) -, healthy adult peripheral blood (PB)-, and myocardial infarct PB-derived EPCs characteristic and surface phenotypes; aim to seek the superior EPCs derived from MNCs for functional improvement of advanced heart failure patient by cell therapy using EPCs.

Materials and Methods

This study was approved by Faculty of Medicine, Universitas Padjadjaran Ethics Committee, Bandung, Indonesia. The UCB were obtained from Graha Bunda Hospital, Bandung with informed consent and approval from the mother, UCB from subjects with questionable health conditions are avoided, for example, difficult deliveries, infectious disease, hepatitis-positive, or sexually transmitted diseases; fresh PB samples were obtained from healthy people and patients with MI obtained from Dr. Hasan Sadikin Hospital were collected and stored in a sterile blood bag containing anti-coagulant at 4°C until processing.

UCBs and PB samples; EPC cultures and conditioned medium (CM) preparation

On receipt of the UCBs or PB samples, it must be handled in an aseptic technique and processed in a Class II biological safety cabinet. MNCs were isolated out of 80–100 ml of UCBs or PB samples by ficoll density-gradient centrifugation (Ficoll Paque Premium GE 17–5442–03), as described previously [10]. These cells were suspended in growth medium consisting of X vivo-15 (Lonza), 20% patient's serum or fetal bovine serum (FBS), 1 ng/ml human recombinant vascular endothelial growth factor (VEGF) (R and D), and 0.1 µmol/L Lipitor atorvastatin (Pfizer). Subsequently, cells were seeded into fibronectin plate. The isolated MNCs were also grown in 96-well plates under identical conditions to be used in the characterization of cells on the harvesting day. On the 7 days after blood collection, the CM was collected and concentrated for the characterization CM. After cell get confluence 80%, adherent cells were detached using 0.5 mmol/L ethylenediaminetetraacetic acid, washed twice, and re-suspended in growth medium.

Acetylated-low-density lipoprotein (Ac-LDL) metabolism as EPCs cell marker molecule

UCB EPCs culture was carried out on 8 wells – micro sterile glass (Matsunami, Japan) which had been coated with type I collagen (Sigma). After reaching confluence, the medium is replaced with a new medium

which has been added Ac-LDL and labeled with fluorescent dye Alexa fluor 488 (green fluorescence, Invitrogen). Subsequent actions, incubated for 4 h at 37°C. Visualization of cells that have metabolized Ac-LDL was observed using confocal laser scanning microscopy (NGO 510 Meta, Zeiss).

Immunofluorescent technique to see EPCs markers

UCB EPCs culture was cultured in sterile culture dishes consisting of 8 wells (Matsunami), then washed 2x with phosphate-buffered saline (PBS). Fixation was carried out with 4% (w/v) cold paraformaldehyde (PFA) for 10 min. EPCs were then washed again with PBS, then added blocking fluid consisting of 10% (v/v) normal serum goat (NGS) and 0.4% (v/v) Triton X-100. Furthermore, the sample was incubated for 30 min at room temperature, then administered primary antibodies for 1 h at room temperature. The antibodies used as marker molecules in this study were: anti-von Willebrand factor (VWF) VIII (vWF, Santa Cruz); anti-CD-31 (platelet endothelial cell adhesion molecule 1 [PECAM-1], BD Pharmingen); and TO-PRO-3 (Molecular Probes). After administration of the first antibody, EPCs were washed again with PBS, the second antibody could be given in PBS (1:1,000 dilution) containing 3% (v/v) NGS and 0.1% (v/v) triton X-100. Incubation is carried out for 30 mins at room temperature. The second antibody used Alexa Flour 488 (green fluorescence, Invitrogen), fluorescein isothiocyanate (FITC) (green fluorescence), and rodhamine (red fluorescence, Jackson Immuno Research). EPCs cells are washed, fixation is carried out with 4% (v/v) PFA for 10 min at room temperature. Fixation results are washed again, then given a slips cover. Specimens that have been made protected using a vector mounting mounting medium (Vector Laboratories) observed using confocal laser scanning microscopy (LSM 510 Meta, Zeiss).

Immunophenotyping and immunocytochemistry

Fluorescence activated cells (FACs) technique was used to determine the typical cell surface epitope profiles for instance CD34, CD45, and CD133. Briefly, early EPCs were incubated with antibodies at 4°C and dark condition 15 min then analyzed by Cell Quest Pro software. As an alternative approach to determine the phenotype of EPCs, immune-fluorescent staining was performed. Cells on 96-well plate were added with Dil-ac-LDL reagent, fixated with 3% PFA, treated UEA-1 Lectin, and subjected to be stained by 4',6-diamidino-2-phenylindole consecutively with appropriate incubation and washing with PBS in-between staining. Fluorescence is observed by microscope fluorescence in the dark condition.

Characterization of blood-derived EPCs-CM

The enzyme-linked immunosorbent assay (Elisa) technique was used to determine the concentration of growth factor VEGF and hepatocyte growth factor (HFG) within the EPC-CM.

Results

Isolation and culture of UCB- and PB-derived EPCs

In our study, EPCs have been succeed to be isolated from human UCB and PB (Figure 1a-c) by Ficoll density-gradient centrifugation. After 7 days of contact with fibronectin-plastic, the detached cell was removed and the medium was replaced every 3 days. Cobble-stone and spindle shape cell were observed under microscope (Figure 1d and e).

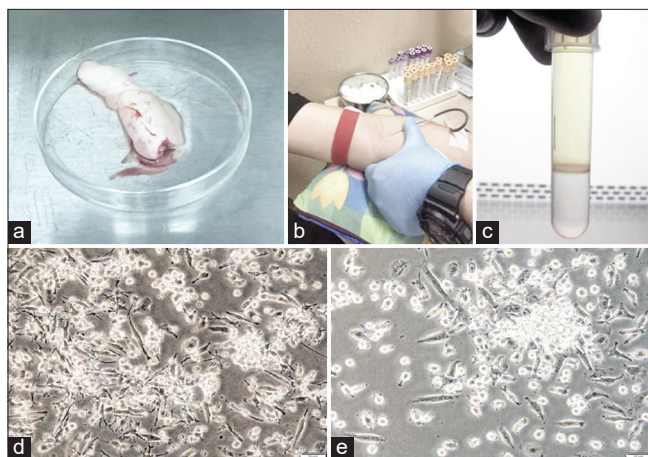


Figure 1: Endothelial progenitor cells have been succeed to be isolated from human umbilical cord blood and peripheral blood by Ficoll density-gradient centrifugation (a-c). After 7 days of contact with fibronectin-plastic, the detached cell was removed and the medium was replaced every 3 days. Cobble-stone and spindle shape cell was observed under microscope (d and e)

Ac-LDL metabolism as EPCs cell marker molecule

The UCB-EPCs were cultured overnight on basement membrane matrix gel, capillary tubule-like structure formation was observed (Figure 2a). The Ac-LDL marker can be used to prove the existence of EPCs cells. Our EPCs shown of Ac-LDL immunoflorescent that looked like green dots evenly distributed in the cell cytoplasm (Figure 2b-d).

Immunoflorescent technique to see EPCs markers

Immunoflorescent clotting factor VIII VWF is a specific marker of endothelial cells (EC), making it a

standard marker molecule for EC in the culture system (Figure 3a); bromodeoxyuridine (BrdU) is used as a proliferation marker molecule. EPCs cells were then stained with immunoflorescent anti-CD31 PECAM-1 and To-Pro-3 molecules calculated in four regions considered representative; the value of viability reached 97–2.3% (Figure 3b).

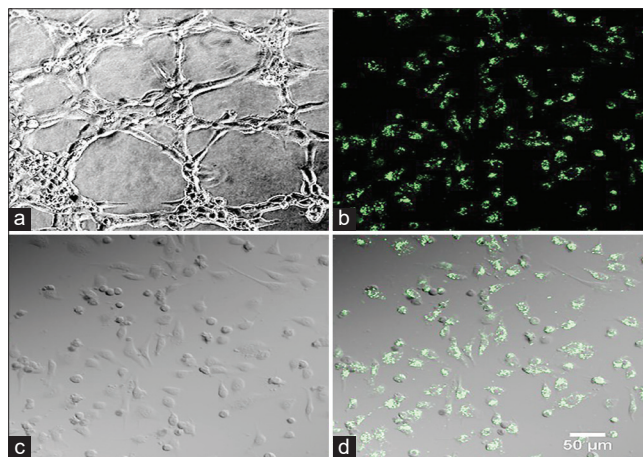


Figure 2: Umbilical cord blood -endothelial progenitor cells (EPCs) were cultured overnight on basement membrane matrix gel, capillary tubule-like structure formation was observed (a). The acetylated-low-density lipoprotein marker was used to prove the existence of EPCs cells. Our EPCs shown of acetylated-low-density lipoprotein immunoflorescent that looked like green dots evenly distributed in the cell cytoplasm (b-d)

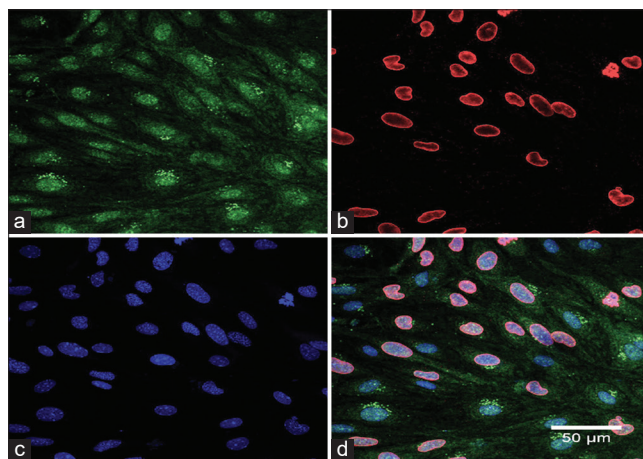


Figure 3: Immunoflorescent clotting factor VIII (vWF) is a standard marker molecule for endothelial cells in the culture system (a); BrdU is used as a proliferation marker molecule. Endothelial progenitor cells were then stained with immunoflorescent anti-CD31 and To-Pro-3 molecules calculated in 4 regions considered representative; the value of viability reached $97 \pm 2.3\%$ (b)

Healthy and MI EPCs by immunophenotyping using FAC and immunohistochemistry

Based on Figure 4a and b, EPCs expressed proteins on their surfaces such as CD34 PE, CD45 FITC, and CD133 as receptors of VEGF. In addition, EPCs exhibited several characters such as have spindle shape morphology on fibronectin matrix,

performed Ac-LDL endocytosis and bind to lectin (Figure 5).

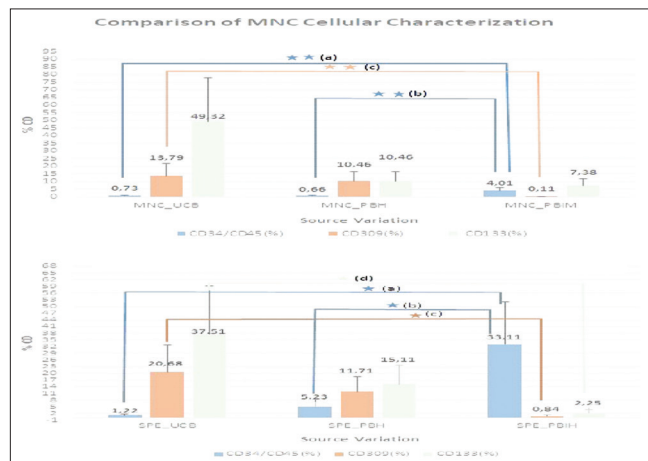


Figure 4: Mononuclear cells and endothelial progenitor cells expressed proteins on their surfaces such as CD34 PE, CD45 FITC, and CD133 as receptors of vascular endothelial growth factor (a and b)

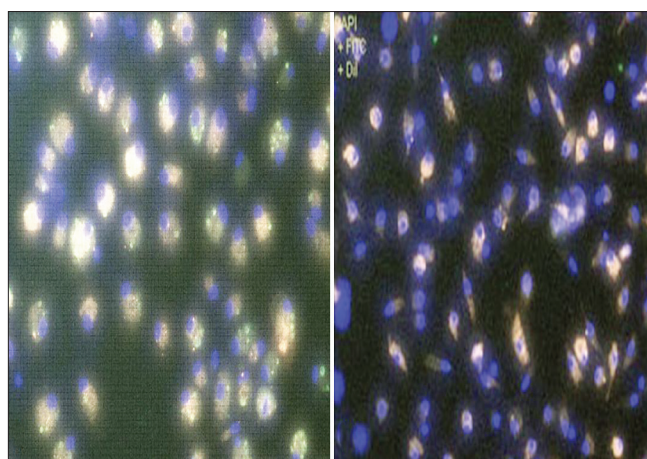


Figure 5: Endothelial progenitor cells exhibited several characters such as spindle shape morphology on fibronectin matrix, performed acetylated-low-density lipoprotein endocytosis and bind to lectin

Characterization of MI, healthy, and UCB-derived EPCs-MNCs

In this study, the characteristic of different isolated sources of each EPCs MNCs as follow: (I) The average MI PB MNCs (PBMNCs) age was 53 year old (YO), the average collected blood volume was 100 cc, the average isolated PB MNCs per ml was 1.165×10^6 with average viability which was 94.2%. (II) The average Healthy PB MNCs age was 27 YO, the average collected blood volume was 90 cc, the average isolated PB MNCs per ml was 1.1×10^6 with average viability was 97.5%. (III) The average UCB-MNCs age was 27 YO, the average collected blood volume was 80 cc, the average isolated MNCs per ml was 5.1×10^6 with average viability which was 96.8%. The measurements of VEGF and HGF in CM-EPC have been done. This study revealed that EPCs produced VEGF and HGF as extracellular growth factor; both concentration in human

PB-derived EPC-CM decreased during culturing. However, VEGF concentration from UCB-derived EPC-CM increased during culturing up to 556 pg/ml using human serum in growth medium and 140 pg/ml using FBS in growth medium. HGF concentration from UCB-derived EPC-CM increased during culturing up to 500 pg/ml using human serum in growth medium and 900–3000 pg/ml using FBS in growth medium.

Discussion

Mesenchymal stem cells and EPCs from human fetal origin are promising sources for regenerative medicine, which has advantage over others resources based in its potential and efficacy [11], [12]. EPCs have been reported, which have role in vasculogenesis, angiogenesis, and re-endothelialization of injured vessels. Recently, study by Xue *et al.* indicates that even low-dose of EPCs transplantation may play a pro-angiogenic, anti-fibroblast, anti-fibrosis, and anti-necrosis role and enhance cardiac function in a rat model [13]. The EPCs angiogenic potential limited by the absence of their natural biologic foundation, namely, smooth muscle pericytes also known as smooth muscle cells (SMCs); EPC-SMCs cell sheet transplantation attenuated myocardial dysfunction [14]. Further, statins treatment also shown augmented both number and function of EPCs [15].

Cord blood (CB) and PB considered as valuable sources of EPCs as they are both easy to obtain with non-invasive procedures [16]. However, isolated EPCs from UCB and adult PBMNCs exhibit different proliferation features and clonogenic potency [17]. In this present study, EPCs were isolated from three different sources, that is, UCB; healthy adult PB and myocardial infarct PB. EPCs can be identified by some characteristic such as expression of surface markers (CD34, CD31, KDR, and VWF) and endothelial functional assays such as endothelial tube formation and Ac-LDL uptake [16]. We observed EPCs obtained from all sources that were growing stably in 2 kinds of growth medium; with FBS and human-serum medium previously. However, there were apparent differences in the number of colonies and the time of appearance between CB-MNCs, PB-MNCs, and MI-MNCs. The number of EPCs-derived CB was 5-fold higher compared with PB-EPCs and MI-EPCs. In agreement with our result, EPCs intrinsically are available in a very low number (<3-5% in the bone marrow and 0.0001-0.01% in the peripheral circulation), therefore need outside intervention to boost their number; development and optimize protocols for their undifferentiated in vitro expansion and manipulation to accentuate their functionality [18].

Nevertheless, 7 days after contact with fibronectin-plastic, EPCs-derived from all sources

configure similar morphology into cobble-stone and spindle shape cells (Figure 1d and e). Furthermore, each EPCs from all sources were also cultured in membrane matrix gel. After overnight incubation, the cells formed capillary tubule-like structure formation. According to Gao *et al.*, [16] one of the main characteristic function of EPCs is capability of cells to incorporate Ac-LDL. EPCs are able to degrade Ac-LDL due to its LDL-receptors without inhibit cell growth. However, the formation of capillary-like structures on Matrigel demonstrating ability to participate in angiogenesis [19]. The other aspect to confirm the authenticity of endothelial cells culture is by detecting VWF expression. VWF is an important component of hemostasis, binding platelets at sites of endothelial damage; it is produced in EC [20]. About 80% of the human umbilical vein EC population culture contain VWF [21].

Anti-CD31 PECAM-1 is also a significant group of endothelial markers. PECAM-1 and CD31 are a 130 kDa protein, which is exclusively expressed and widely distributed on EC [22]. PECAM-1 is heavily glycosylated transmembrane homophilic adhesion protein that is required for migration of leukocytes, playing a key role in removing aged neutrophils from the body and stabilize blood circulation [23]. The extracellular domain of CD31 is released during apoptosis of EC. This fragment circulates in the serum of patients suffering from MI [24]. In addition, BrdU and To-Pro-3 were used to observe proliferation activity of EPCs. The value of viability reached $97\% \pm 2.3\%$ (Figure 3b). In other words, no significant differences were identified between CB-EPC, PB-EPC, and MI-EPC either in endothelial functional assays such as endothelial tube formation and Ac-LDL uptake or in the expression of surface markers (CD34, CD133, CD45, and VWF). The differentiation capacity of progenitor cells is reduced in heart disease patients, which may hamper the clinical efficacy of autologous stem cell therapy; progenitor cells from outside patient body should be added to increase the success rate of cell therapy [25].

On the other hand, there are peculiar differences between the level of VEGF and HGF in CM of CB-EPC. This study revealed that EPCs-derived from CB which is cultured in human serum produced higher concentration of VEGF than those which cultured in FBS. VEGF or vascular permeability factor was originally described as an EC-specific mitogen [26]. VEGF also induces the formation of the Willebrand factor and thrombogenesis and promotes collateral formation in the ischemic myocardium through the following mechanisms such as stimulates EC proliferation, regulates of several endothelial integrin receptors during sprouting of new blood vessels, and microvascular permeability [27], [28]. The potential of human serum as a substitute of FBS has been recognized for decades. However, human serum performed better activities in stimulating proliferation in several cells including EC [19]. We hypothesized that the increasing level of VEGF in human serum-CB-EPC is correlated with the escalation proliferation activity.

Meanwhile, endothelial produced higher HGF when cells were cultured in FBS. HGF is a mesenchyme-derived mitogen that also stimulates cell migration and mediate mesenchymal-epithelial and endothelial interactions, which contribute to tissue repair [29]. The mechanism underlying the differences between level concentration of HGF and VEGF produced by UCB-EPCs was remain unclear.

Conclusion

Our study demonstrated that the superior sources of EPCs for cardiovascular disease therapy is UCB due to the large-number of cell harvested and the extracellular growth factor such as VEGF and HGF in CM-EPCs is higher compare the PB.

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