Proliferation of Peripheral Blood-derived Endothelial Progenitor Cells from Stable Angina Subjects

Yudi Her Oktaviono1,2, Djanggan Sargowo2,3, Mohammad Aris Widodo2,4, Yanni Dirgantara5, Angliana Chouw5, Ferry Sandra6,7,8,9,*

1Department of Cardiology and Vascular Medicine, Dr. Soetomo Hospital, Faculty of Medicine, University of Airlangga, Jl. May. Jend. Prof. Dr. Moestopo No.6-8, Surabaya, Indonesia
2Postgraduate Program in Biomedics, Faculty of Medicine, Brawijaya University, Jl. Veteran, Malang, Indonesia
3Department of Cardiology and Vascular Medicine, Dr. Saiful Anwar General Hospital, Faculty of Medicine, Brawijaya University, Jl. Jaksa Agung Suprapto No. 2, Klojen, Malang, Indonesia
4Department of Pharmacology, Faculty of Medicine, Brawijaya University, Jl. Veteran, Malang, Indonesia
5Prodia Stem Cell Laboratory, Jl. Kramat 7 No.11, Jakarta, Indonesia
6Department of Biochemistry and Molecular Biology, Faculty of Dentistry, Trisakti University, Jl. Kyai Tapa No.260, Jakarta, Indonesia
7Postgraduate Program in Biomedics, Faculty of Dentistry, Trisakti University, Jl. Kyai Tapa No.260, Jakarta, Indonesia
8BioCORE Laboratory, Faculty of Dentistry, Trisakti University, Jl. Kyai Tapa No.260, Jakarta, Indonesia
9Prodia Clinical Laboratory, Prodia Tower, Jl. Kramat Raya No.150, Jakarta, Indonesia
*Corresponding author. E-mail: ferrysandra@gmail.com

BACKGROUND: A population of circulating Endothelial Progenitor Cells (EPCs) has been reported to play important role in maintaining endothelial function and integrity. Since EPCs culture is crucial and an optimized medium is currently available. Therefore we conducted a study to investigate whether stable angina subjects peripheral blood-derived EPCs could be cultured in this medium. Here, we performed study to detect EPCs characteristics and extracellular signal-regulated kinase (Erk)1/2 Mitogen-Activated Protein Kinase (MAPK) pathway as possible underlying pathway for EPCs proliferation.

METHODS: Peripheral blood EPCs from 8 stable angina subjects were cultured in an optimized medium with/without addition of supplement for 1 or 3 days. Then, the membrane of cultured EPCs were detected with immunofluorescence method for CD34, Vascular Endothelial Growth Factor Receptor 2 (VEGFR-2) and CD133. Colony forming unit (CFU) enumeration was performed. XTT Cell proliferation assay was performed to assess EPCs growth after 1 and 3-days culture. The western blot analysis was performed to detect possible activation of Erk1/2 MAPK.

RESULTS: Number of EPCs and CFU cultured for 3 days were significantly higher than the ones cultured for 1 day.
Cardiovascular disease (CVD) is the main cause of mortality in developed and developing countries.\(^1\) Multiple cardiovascular risk factors cause endothelial injury and endothelial dysfunction leading to vasoconstriction, thrombosis, infiltration of monocyte cells, smooth muscle cell proliferation, and formation of atherosclerosis.\(^2\,3\) Maintenance of endothelial integrity and function are important to preserve a healthy vasculature.\(^4\) Indeed, the balance between endothelial injury and endothelial recovery is paramount to reduce cardiovascular events.\(^5\)

Many studies have identified a population of circulating Endothelial Progenitor Cells (EPCs) integrating into sites of neovascularization and endothelial impairment.\(^6\) Circulating EPCs are mobilized immature cells from bone marrow into the bloodstream, in response to particular growth factors and cytokines. EPCs may contribute in vascular repair after differentiate into endothelial cell.\(^7\)

Clinical studies showed that risk factors of atherosclerosis are related to the reduced levels of circulating EPCs.\(^2\,7\) The functional integrity of the endothelium is also comparable to the activities of EPCs.\(^2\) The amount of circulating EPCs correlates negatively with the established risk.\(^8\) Decreased level of circulating EPCs has become an independent predictors of atherosclerotic disease progression and measurement of EPCs has become a predictive value for cardiovascular outcomes in stable coronary artery disease patients.\(^4\,9\) The reduced circulating EPCs can reflect the potential cardiovascular morbidity and mortality.\(^10\,12\) A long-term study shows that assessment of subpopulations of circulating EPCs in patients with stable angina treated with percutaneous coronary intervention can improve characterization of long-term prognosis, suggesting the possibility of using EPCs as biomarkers for the prediction of cardiovascular outcome.\(^13\)

In regards to mechanisms and underlying signaling pathways, spleen-derived EPCs proliferation was shown to be regulated by the phosphatidylinositol-3-kinase (PI3K)/Akt/nuclear factor kappa B (NFκB)/survivin signaling pathway.\(^14\) Meanwhile, in liver regeneration, Notch signaling differentially regulated bone marrow-derived two types of EPCs, early EPCs and endothelial outgrowth cells.\(^15\) Transforming Growth Factor beta 1 (TGF-β1) was reported to play a role in the growth and differentiation of EPCs. Involvement of extracellular signal-regulated kinase (Erk)1/2 Mitogen-Activated Protein Kinase (MAPK) signaling pathway was shown in Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF)-induced peripheral blood-derived EPCs culture.\(^16\) Since EPCs culture is crucial, and an optimized mediums is currently available. This medium could be potential to accelerate EPCs proliferation. However, by using this medium, characteristics, proliferation and signaling pathway of cultured-EPCs derived from different sources, are not disclosed yet. In our current study, stable angina subjects peripheral blood-derived EPCs were cultured in an established culture medium, and then the EPCs characteristics and Erk1/2 MAPK activation were investigated.
Methods

Sample Collection and Mononuclear cells (MNCs) Isolation
Upon signing informed consent, peripheral blood was collected from 8 volunteers with inclusion criteria as follows: stable angina pectoris, males, 50-55 years old, coronary angiogram showing >50% stenotic lesions. Subjects with history of coronary stent, acute myocardial infarction, diabetes mellitus, smoking, critical limb ischemia, or coronary artery bypass graft surgery were excluded from this study. This protocol was approved by The Ethical Committee Medical Research Medical Faculty Brawijaya University (No.273/EC/KEPK-S3/06/2013) and The Ethical Committee / Institutional Review Board of National Cardiac Center Harapan Kita Hospital (No. LB.05.01.1.4/55/2013). MNCs was isolated using Lymphoprep (Stemcell Technologies, Vancouver, Canada). Briefly, collected peripheral blood was immediately diluted with equal amount of phosphate buffer saline (PBS) plus 2% fetal bovine serum (FBS), then layered on top of Lymphoprep. With centrifugation at 800 x g for 20 minutes, MNCs layer was formed and collected.

EPCs Culture
Colony Forming Unit (CFU)-Hill Liquid Medium Kit (Stemcell Technologies) was used for EPCs culture. Briefly, after rinsed in PBS plus 2% FBS, MNCs were suspended in CFU-Hill Liquid Medium. In each well of 6-well plate, 5x10^6 cells of MNCs suspension was plated and incubated for 2 days in humidified 37°C 5% CO2 incubator. Then, non-adherent cells were harvested and further cultured for 3 days to allow formation of CFU-Hill colonies. CFU-Hill colonies were enumerated according to kit insert.

Immunofluorescence
After rinsed with PBS, cells were fixed with 4% paraformaldehyde. Fixed cells were then treated with 100 mM glycine and 0.2% Triton X-100 for permeabilization. To block nonspecific binding, 0.1% bovine serum albumin in PBS was applied. For antibodies probing, a combination of 1:400 diluted mouse monoclonal Alexa Fluor 488 anti-human CD34, 1:200 diluted mouse monoclonal R-phycoerythrin-conjugated anti-CD309 (VEGFR-2/KDR) and 1:180 diluted rabbit polyclonal anti-CD133 antibodies, was applied. Then 1:1000 diluted Alexa Fluor 350 F(ab")2 fragment of goat anti-rabbit IgG (H+L) was applied as a secondary antibody for the anti-CD133 antibody. To have better cell determination, 4',6-diamidino-2-phenylindole (DAPI) staining was performed. Cell expression was documented under a fluorescence microscope.

Cell Proliferation Assay
Na,3'-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate (XTT) Cell Proliferation Assay Kit (Cayman Chemical Company, Ann Arbor, MI) was used for cell proliferation assay. The assay is based on the extracellular reduction of XTT by nicotinamide adenine dinucleotide (NADH) produced in the mitochondria via transplasma membrane electron transport and an electron mediator. Briefly, XTT mixture was added into each well and incubated in a 37°C 5% CO2 incubator for 4 hours. The absorbance was measured using a microplate reader at a wavelength of 450 nm. To interpolate cell number, some wells containing cells were trypsinized and stained with trypan blue. The cell was then counted using hemocytometer under an inverted light microscope.

Western Blot
Cells were harvested and incubated with lysis buffer containing 25 mM Tris HCl buffer (pH 7.6), 150 mM NaCl, 1% Triton-X, 0.1% sodium dodecyl sulfate (SDS), Protease Inhibitor Cocktail [containing 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, aprotinin, bestatin hydrochloride, N-(trans-Epoxysuccinyl)-L-leucine 4-guanidinobutylamide, leupeptin, pepstatin A] (Sigma-Aldrich, St.Louis, MO]. Phosphatase Inhibitor Cocktail 3 [containing cantharidin, (-)-p-bromolevamisole oxalate, calyculin A (Sigma-Aldrich)]. Samples were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Inc., Hercules, CA). After blocking with 5% skim milk in PBS, the membrane was probed with 1:1000 diluted rabbit polyclonal anti-phospho-Erk1/2 MAPK (Thr202/Tyr204) antibody (Cell Signaling Technology, Inc., Danvers, MA). The secondary antibody was 1:2000 diluted goat anti-rabbit IgG horseradish peroxidase-linked antibody (Cell Signaling Technology, Inc.). The bound antibodies were visualized using Immun Star HRP Chemiluminescent Kit (Bio-Rad Laboratories, Inc.). Membrane was then stripped with Seppro stripping buffer (Sigma-Aldrich), blocked with 5% skim milk in PBS, probed with rabbit polyclonal anti-Erk1/2 MAPK (Cell Signaling Technology, Inc.), bound with same secondary antibody and visualized with chemiluminescent kit. All visualized bands were captured using Alliance 4.7 (UViTech, Ltd., Cambridge, UK) and quantified using UViBand software (UViTech, Ltd.).
Results

Peripheral blood-derived EPCs Membrane Marker Expression
EPCs membrane markers of CD34 (Figure 1A), VEGFR2 (Figure 1B) and CD133 (Figure 1C) were expressed under a fluorescent microscope. EPCs with CD34+, VEGFR2+ and CD133+ expressions were merged (Figure 1D). The captured EPCs were shown as a CFU under a light microscope (Figure 1E).

Increased number of EPCs and CFU
Number of EPCs and CFU cultured for 3 days were significantly higher than the ones cultured for 1 day.

Statistical Analysis
Analyses were performed using IBM SPSS for Windows version 19.0 (IBM Corp., Armonk, NY). Wilcoxon Signed Rank Test was used to determine the statistical differences. A probability value <0.05 was considered to be statistically significant.

Figure 1. Expression of EPCs membrane markers and CFU. Collected MNCs were cultured and characterized with immunofluorescence as described in Methods. EPCs membrane markers of CD34 (A), VEGFR2 (B) and CD133 (C) were documented. In addition, DAPI-stained EPCs were also visualized (C) under an inverted fluorescence microscope. D: Merge of A, B & C. E: CFU-Hill colony was documented under an inverted light microscope. White bar: 50 μm. Black bar: 100 μm.

Figure 2. Supplement induced EPCs proliferation and CFU formation.
After collected MNCs were incubated for 2 days as described in Methods, the non-adherent cells were harvested and cultured for 1 or 3 days with supplement of medium kit. For EPCs proliferation, XTT assay was performed as described in Methods. Meanwhile, CFU was enumerated under a standard light microscope. Data were analyzed with Wilcoxon Signed Rank Test, *p<0.05. (p=0.012) (Figure 2). Number of EPCs was increased significantly at 1.94-fold (p=0.012). In the same comparison between culture of 3 days and 1 day, number of CFU was increased significantly at 2.36-fold (p=0.012).

Erk2 MAPK activation in EPCs derived from stable angina subjects
Expressions of Erk1/2 MAPK were observed in EPCs derived from stable angina subjects treated with/without supplement, a part of CFU-Hill Liquid Medium Kit, and/or U0126, a MAPK Erk1/2 (MEK1/2) inhibitor, for 1 or 3 days (Figure 3). Meanwhile phosphorylated-Erk2 expression of stable angina subjects was slightly observed only in EPCs treated with supplement for 3 days. Number of viable EPCs was significantly increased for the group treated with supplement than without supplement in both durations of 1...
There is an increasing interest in EPCs due to their contribution in the maintenance of endothelial integrity. (5) At first, the description of EPCs isolation from peripheral blood was introduced by Asahara et al. (17) Then later on, the evolving process of EPCs into mature and functional endothelial cells was reported. (8,18) In this current study, we aim to analyze the cultured-EPCs derived from stable angina subjects in an established commercially available medium. Peripheral blood-derived EPCs were generated and confirmed by verifying EPCs membrane marker CD34, CD133 and VEGFR2. In addition, CFUs were also resulted. We found that the number of EPCs and CFUs significantly increased upon addition of supplement provided along with medium (p=0.012). When we cultured EPCs in a longer duration for 3 days, higher numbers of EPCs and CFUs were observed.

**Discussion**

MAPK pathway relies, amplifies and integrates signals from any enormous array of stimuli and establish some physiological responses, such as: cellular proliferation, differentiation, development, transformation, inflammatory response and apoptosis. (19,20) Moreover, it regulates gene expression, metabolism, cell division, morphology, and survival as well as proteins phosphorylation. (20) There are 3 common subfamilies of MAPK: Erk, Jun kinase (Jnk) and p38 MAPK in which Erk MAPK has been the most extensively studied subfamily by far. (19,21) Mitogenic stimulation by some particular growth factors may cause activation of the classical Erk family (ERK1/p44 or ERK2/p42 MAPK). (19,21-23) It has been hypothesized that the activation of MAPK at G1 seems to be related with its ability to enter S phase. (19) Since involvement of Erk1/2 MAPK...
signaling pathway was reported in peripheral blood-derived EPCs culture (16), we then pursued our study in Erk1/2 signaling pathway as potential pathway for the supplement to induce EPCs proliferation and CFUs formation.

Our current results showed that under induction of supplement, Erk2 MAPK of EPCs derived from stable angina subjects were slightly phosphorylated. In contrast, by addition of U0126, phosphorylation of Erk2 was totally diminished. Along with the Erk2 phosphorylation inhibition, EPCs growth and CFU formation were also inhibited by U0126, showing that activated pathway was ERK2 MAPK-dependent.

Conclusion

Taken together, our results showed that an important role of p42 MAPK in supplement-induced peripheral blood EPCs derived from stable angina subjects. Further investigation in component of supplement and other potential activated signaling pathway should be carried out to clarify the mechanism of EPCs proliferation induction in this medium.

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