Biochemical and Biophysical Research Communications xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# Smooth muscle progenitor cells from peripheral blood promote the neovascularization of endothelial colony-forming cells

Hyung Joon Joo [a], Ha-Rim Seo [a], Hyo Eun Jeong [b], Seung-Cheol Choi [a], Jae Hyung Park [a], Cheol Woong Yu [a], Soon Jun Hong [a], Seok Chung [b], Do-Sun Lim [a,*]

[a] Department of Cardiology, Cardiovascular Center, College of Medicine, Korea University, Seoul, Republic of Korea
[b] Department of Mechanical Engineering, Korea University, Seoul, Republic of Korea

ARTICLE INFO

*Article history:*
Received 7 May 2014
Available online xxxx

*Keywords:*
Smooth muscle progenitor cell
Endothelial-colony forming cell
Adult peripheral blood
Neovascularization
Ischemic hindlimb

ABSTRACT

Proangiogenic cell therapy using autologous progenitors is a promising strategy for treating ischemic disease. Considering that neovascularization is a harmonized cellular process that involves both endothelial cells and vascular smooth muscle cells, peripheral blood-originating endothelial colony-forming cells (ECFCs) and smooth muscle progenitor cells (SMPCs), which are similar to mature endothelial cells and vascular smooth muscle cells, could be attractive cellular candidates to achieve therapeutic neovascularization. We successfully induced populations of two different vascular progenitor cells (ECFCs and SMPCs) from adult peripheral blood. Both progenitor cell types expressed endothelial-specific or smooth muscle-specific genes and markers, respectively. In a protein array focused on angiogenic cytokines, SMPCs demonstrated significantly higher expression of bFGF, EGF, TIMP2, ENA78, and TIMP1 compared to ECFCs. Conditioned medium from SMPCs and co-culture with SMPCs revealed that SMPCs promoted cell proliferation, migration, and the *in vitro* angiogenesis of ECFCs. Finally, co-transplantation of ECFCs and SMPCs induced robust *in vivo* neovascularization, as well as improved blood perfusion and tissue repair, in a mouse ischemic hindlimb model. Taken together, we have provided the first evidence of a cell therapy strategy for therapeutic neovascularization using two different types of autologous progenitors (ECFCs and SMPCs) derived from adult peripheral blood.



## 1. Introduction

Neovascularization is a process essential to improving tissue ischemia and restoring organ regeneration. During the last few decades, cell therapy using stem and progenitor cells has been extensively utilized to achieve therapeutic neovasculogenesis.

Among various stem and progenitor cells, endothelial colony-forming cells (ECFCs) deserve extra attention for their neovasculogenic potential [1,2]. ECFCs are also known as late-outgrowth endothelial cells, blood-derived outgrowth endothelial cells, or endothelial progenitor cells and are a blood-originating endothelial cell-like population that possesses similar gene expression and function to mature endothelial cells. ECFC implantation has been demonstrated to induce de novo neovessels *in vivo* and improve functional recovery in ischemic disease models [3,4]. Although ECFCs have been thought to have comparable neovasculogenic potential to mature endothelial cells, one report demonstrated that that ECFCs have inferior *in vivo* neovasculogenic potential compared to mature endothelial cells [5]. Therefore, many attempts to modulate ECFC activity have been also reported. Priming with growth factors or cytokines [6,7], gene transfer [8], and combined transplantation with other cell types [9,10] have all been proposed to promote the neovasculogenic potential of ECFCs *in vitro* and *in vivo*. In addition to ECFCs, smooth muscle progenitor cells (SMPCs) can also be induced from human blood [11]. SMPCs have been suggested to be associated with atherosclerosis [12]. However, little has been studied regarding other functions of SMPC in neovasculogenesis.

Considering that (1) vessels contain both endothelial cells and vascular smooth muscle cells, (2) neovasculogenesis is the harmonized cellular response of both cell types, both ECFCs and SMPCs might have roles during *in vivo* neovasculogenesis. Moreover, it is clinically feasible to induce both cell types from adult peripheral blood. In the present study, we characterized both ECFCs and SMPCs and tested the hypothesis that transplanted ECFCs and SMPCs synergistically contribute to new vessel formation and improve tissue regeneration after ischemic injury.

* Corresponding author. Address: Department of Cardiology, Cardiovascular Center, Korea University Anam Hospital, 126-1, 5ka, Anam-dong, Sungbuk-ku, Seoul 136-705, Republic of Korea. Fax: +82 2 927 1478.
*E-mail address:* dslmd@kumc.or.kr (D.-S. Lim).

http://dx.doi.org/10.1016/j.bbrc.2014.05.061
0006-291X/

## 2. Methods

### 2.1. Cell culture

The Institutional Review Board at the Korea University Anam Hospital approved the study protocols. Adult peripheral blood samples were obtained from volunteers after informed consent was obtained. ECFCs and SMPCs were induced as described previously [11,13]. Briefly, mononuclear cells (MNCs) were separated from blood using Ficoll-Paque (GE-Healthcare). Then, $8.0 \times 10^6$ MNCs were plated on collagen-coated 12-well plates (Stem Cell Technologies) and cultured in EGM-2MV (Lonza). For SMPC induction, we supplemented PDGF-BB (50 ng/mL) into EGM-2MV. Purities of ECFCs and SMPCs were defined by cell morphology, immunofluorescent staining, and flow cytometric analysis. Mixtures of ECFCs and SMPCs were not used for the experiments. Normal karyotyping of the adult peripheral blood-derived ECFCs and SMPCs was confirmed (data not shown).

### 2.2. Fibrinogen bead assay

Cytodex3™ microcarrier beads (GE Healthcare) were hydrated according to the manufacturer's protocol. Briefly, microbeads were swollen for 3 h in PBS, washed, resuspended in PBS, and then autoclaved at 121 °C for 15 min. ECFCs ($1.0 \times 10^6$) and microbeads ($2.5 \times 10^3$) were suspended in EGB2-MV medium and incubated for 4 h with gentle agitation at 15-min intervals. The cell-covered microbeads were mixed in fibrinogen (2 mg/mL, Sigma) and the fibrinogen was then polymerized with thrombin (0.625 U/mL, Sigma). Culture medium was added into the polymerized fibrinogen. Sprouting angiogenesis was analyzed 4 days following the addition of the culture medium.

### 2.3. In vivo Matrigel plug assay

Cells were mixed with 100 μL Matrigel, and the mixture was implanted subcutaneously into the dorsal side of eight-week-old nude/SCID mice. After 2 weeks, the mice were sacrificed under anesthesia (intramuscular injection of 80 mg/kg ketamine and 12 mg/kg xylazine), and the implanted Matrigels were harvested for histologic analyses.

### 2.4. Ischemic hindlimb model

All animal care and experimental procedures were performed in accordance with the Guidelines for Animal Care and Use of the Korea University School of Medicine. All protocols were approved by the Administrative Panel on Laboratory Animal Care at the Korea University School of Medicine. Eight-week-old nude/SCID mice underwent surgical ligation of the proximal part of the left femoral artery under anesthesia. Cells were then injected intramuscularly into three sites of the hindlimb adductor muscle. Laser Doppler perfusion images (Moor Instruments, Devon, United Kingdom) were assessed at days 3, 7, 14, 21, and 28. The hindlimb adductor muscle and contralateral adductor muscle were harvested for histologic analyses at day 28.

### 2.5. Immunofluorescence staining, flow cytometric analysis, semi-quantitative RT-PCR, Matrigel tube-forming assay, Angiogenic cytokine array, cell viability assay, in vitro scratch wound healing assay, adipogenic and osteogenic differentiation

These procedures were performed as described in detail in the Supplementary methods.

### 2.6. Statistics

Values are presented as means ± standard deviation (SD). Significant differences between the means were determined by analysis of variance followed by the Student–Newman–Keuls test. Significance was set at $p < 0.05$.

## 3. Results

### 3.1. Characterization of ECFCs and SMPCs derived from adult peripheral blood

Based on previous literatures [11,13], we successfully induced both ECFC and SMPC derivation from adult peripheral blood (Fig. 1A). ECFCs had a typical cobblestone appearance, similar to mature endothelial cells, and SMPCs were spindle-shaped cells with a pronounced hill and valley pattern when they were confluent (Fig. 1B).

Immunofluorescent staining demonstrated that ECFCs expressed typical endothelial junctional markers, CD31 and CD144, and did not express smooth muscle markers such as vimentin, desmin and αSMA (Fig. 1C). On the contrary, SMPCs did not express CD31 and VE-cadherin, and expressed vimentin, desmin and αSMA.

Flow cytometric analyses also demonstrated that both ECFCs and SMPCs did not express hematopoietic (stem) cell markers (Lineage, CD45, CD117, CD133) (Fig. 1D). Endothelial cell markers (CD144, CD31, Tie2, KDR) were expressed in ECFCs but not in SMPCs. Both ECFCs and SMPCs expressed CD105, which is reported to be expressed in SMPCs as well as in ECFCs [14]. RT-PCR also confirmed the higher expression of endothelial cell-related genes (CD144, vWF, Angiopoietin-2 [Ang2]) in ECFCs and the higher expression of smooth muscle cell-related genes (αSMA, SM22α, Calponin) in SMPCs (Fig. 1E). Moreover, the ECFCs showed typical endothelial cell function, such as Matrigel-induced tubular network formation (Fig. 1F).

### 3.2. Differential angiogenic cytokine expression in ECFC and SMPC

To compare the expression of angiogenic cytokines and growth factors between ECFCs and SMPCs, a total of 43 angiogenesis-related growth factors and cytokines were analyzed using the angiogenic cytokine array. Angiogenic cytokine array analysis showed that ECFCs and SMPCs have different angiogenic cytokine expression profiles (Fig. 1G). ECFCs expressed EGF, bFGF, GRO, IL-8, MCP1, TIMP1, TIMP2, Ang2, Endostatin, IL-4, MMP1, MMP9, PECAM1, Tie2, μPAR, and VEGFR2. SMPCs expressed EGF, ENA78, bFGF, GRO, IL-8, TIMP1, TIMP2, and μPAR. When the relative expressions were compared between ECFCs and SMPCs, bFGF (2.6-fold), EGF (17-fold), TIMP2 (2.5-fold), ENA78 (not detected in ECFC), and TIMP1 (5.6-fold) revealed higher expression levels in SMPCs, while GRO (2.0-fold), Ang2 (not detected in SMPC), μPAR (2.5-fold), MCP1 (16-fold), and PECAM1 (not detected in SMPC) revealed higher expression levels in ECFCs (Fig. 1H).

### 3.3. SMPCs enhanced ECFC proliferation, migration, and angiogenic sprouting in vitro

Considering that ECFCs and SMPCs have different angiogenic cytokine expression patterns, we hypothesized that SMPCs could promote the cell proliferation, migration, and angiogenic potential of ECFCs through their complementary angiogenic cytokine expression. We compared the effect of SMPC-conditioned medium (CM) on ECFC proliferation and migration to those of control medium (Control, EGM-2MV) and ECFC-CM. SMPC-CM increased

Please cite this article in press as: H.J. Joo et al., Smooth muscle progenitor cells from peripheral blood promote the neovascularization of endothelial colony-forming cells, Biochem. Biophys. Res. Commun. (2014), http://dx.doi.org/10.1016/j.bbrc.2014.05.061