Journal of Molecular and Cellular Cardiology 86 (2015) 121–135

Contents lists available at ScienceDirect

Journal of Molecular and Cellular Cardiology

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

Original article

Diverse contribution of bone marrow-derived late-outgrowth endothelial progenitor cells to vascular repair under pulmonary arterial hypertension and arterial neointimal formation

Masayasu Ikutomi [a,b], Makoto Sahara [a,c,*], Toshiaki Nakajima [a], Yoshiyasu Minami [a], Toshihiro Morita [a], Yasunobu Hirata [a], Issei Komuro [a], Fumitaka Nakamura [b], Masataka Sata [d]

[a] Department of Cardiovascular Medicine, University of Tokyo Graduate School of Medicine, Tokyo 113-8655, Japan
[b] Department of Cardiology, Teikyo University Chiba Medical Center, Chiba 299-0111, Japan
[c] Department of Medicine-Cardiology/Cell and Molecular Biology, Karolinska Institutet, SE-171 77 Stockholm, Sweden
[d] Department of Cardiovascular Medicine, Institute of Health Biosciences, The University of Tokushima Graduate School of Medicine, Tokushima 770-8503, Japan

ARTICLE INFO

Article history:
Received 13 March 2015
Received in revised form 10 July 2015
Accepted 24 July 2015
Available online 29 July 2015

Keywords:
Endothelial progenitor cell
Monocrotaline
Post-angioplasty restenosis
Pulmonary arterial hypertension
Vascular disease

ABSTRACT

Aims: It is still controversial whether bone marrow (BM)-derived endothelial progenitor cells (EPCs) can contribute to vascular repair and prevent the progression of vascular diseases. We aimed to characterize BM-derived EPC subpopulations and to evaluate their therapeutic efficacies to repair injured vascular endothelium of systemic and pulmonary arteries.

Methods and results: BM mononuclear cells of Fisher-344 rats were cultured under endothelial cell-conditions. Early EPCs appeared on days 3–6. Late-outgrowth and very late-outgrowth EPCs (LOCs and VLOCs) were defined as cells forming cobblestone colonies on days 9–14 and 17–21, respectively. Among EPC subpopulations, LOCs showed the highest angiogenic capability with enhanced proliferation potential and secretion of proangiogenic proteins. To investigate the therapeutic effects of these EPCs, Fisher-344 rats underwent wire-mediated endovascular injury in femoral artery (FA) and were concurrently injected intraperitoneally with 60 mg/kg monocrotaline (MCT). Injured rats were then treated with six injections of one of three EPCs (1×10^6 per time). After 4 weeks, transplanted LOCs, but not early EPCs or VLOCs, significantly attenuated neointimal lesion formation in injured FAs. Some of $CD31^+$ LOCs directly replaced the injured FA endothelium (replacement ratio: $11.7 \pm 7.0\%$). In contrast, any EPC treatment could neither replace MCT-injured endothelium of pulmonary arterioles nor prevent the progression of pulmonary arterial hypertension (PAH). LOCs modified protectively the expression profile of angiogenic and inflammatory genes in injured FAs, but not in MCT-injured lungs.

Conclusion: BM-derived LOCs can contribute to vascular repair of injured systemic artery; however, even they cannot rescue injured pulmonary vasculature under MCT-induced PAH.

1. Introduction

Cumulative evidence suggests that bone marrow (BM)-derived endothelial progenitor cells (EPCs) play an important role in the repair of injured vascular endothelium and in postnatal angiogenesis [1,2]. EPCs are recruited from the bone marrow to injury foci, where they differentiate into mature endothelial cells that are capable of engrafting with the native vessels. Regeneration of the injured vascular endothelium by transplantation of the ex vivo expanding autologous EPCs can be a novel therapeutic approach for ischemic cardiovascular diseases [2,3]. Indeed, several investigators have shown that EPC treatment had efficacy in therapeutic revascularization/reendothelialization by their direct engrafting and paracrine effects, following the improvement of the blood flow and function of ischemic tissues/organs, in various vascular disease models of the experimental [4–6] and clinical [7,8] settings, although other groups have reported conflicting results questioning the contribution of EPCs to vascular repair and angiogenesis [9,10]. The discrepancies in therapeutic potential of EPCs reported in these studies may be attributable to differences in the definition of EPCs and/or in the methods of isolation and expansion of EPCs, at least in part. A recent development in the characterization of human peripheral blood mononuclear cell (hPBMNC)-derived EPCs has identified the two major cell types of EPCs, named as early EPCs and late-outgrowth EPCs [11–13]. Early EPCs appearing in an early phase of culture are similar to those originally described by Asahara et al. [1] and have been previously used in a great number of experimental EPC studies. Early EPCs are identified as $CD34^+$/vascular endothelial growth factor receptor (VEGFR2, also known as Flk-1)$^+$ cells, also having a pan-leukocyte surface marker

* Corresponding author at: Department of Medicine-Cardiology/Cell and Molecular Biology, Karolinska Institutet, SE-171 77 Stockholm, Sweden.
E-mail address: makoto.sahara@ki.se (M. Sahara).

http://dx.doi.org/10.1016/j.yjmcc.2015.07.019
0022-2828/

CD45 [13,14]. In contrast, late outgrowth EPCs emerge in a later phase of culture and form colonies with cobblestone appearance, along with higher proliferative potential and the phenotype more specific to endothelial cells [13,14]. Recent studies have indicated the functional differences between the two EPCs. Early EPCs have a limited potential to engraft and incorporate into newly-formed vessels in ischemic tissue irrespective of their proangiogenic effects by secretion of angiogenic factors such as vascular endothelial growth factor (VEGF), whereas late-outgrowth EPCs show superior angiogenic potential for revascularization by both direct engrafting and paracrine effects [13–16]. However, these findings are basically derived from the studies investigating hPBMNC-derived EPCs, and the detailed classification and characterization of rodents or human BM-derived EPCs remains to be fully determined.

Vascular endothelial injury and dysfunction can trigger not only systemic arteriosclerosis including post-angioplasty restenosis, but also pulmonary arterial hypertension (PAH) [17–19]. PAH is a progressive fatal disorder with a poor prognosis irrespective of the recent development of treatment strategies, and the exact pathogenesis of PAH is still uncertain [18,19]. Because endothelial damage and dysfunction in pulmonary arteries is believed to be one of the key pathological events of PAH, EPCs have been investigated so far to evaluate their therapeutic potential for PAH. However, there is still no consensus about whether EPCs can substantively contribute to vascular repair and prevent the disease progression of PAH: some experimental studies have suggested that the transplantation of exogenous EPCs might hold therapeutic promise for PAH [20–22], whereas others have doubted the contribution to vascular repair and therapeutic potential of EPCs [23–25].

On this basis, we first determined the in vitro classification and characterization of rat BM-derived EPCs, including early EPCs, late-outgrowth EPCs (LOCs) and very late-outgrowth EPCs (VLOCs), which were defined by the timings of emergence and culture periods, respectively. Then, the in vivo therapeutic effects of the three EPCs to potentially contribute to the restoration of injured systemic and/or pulmonary arterial endothelium were examined using a simultaneous injury model in rats [26]: monocrotaline (MCT)-induced PAH and wire-mediated endovascular injury in femoral artery (FA). Here we show that among the three BM-derived EPC subpopulations, LOCs had the highest angiogenic potential with enhanced proliferative property and secretion of angiogenic proteins such as VEGF and basic fibroblast growth factor (FGF), and that only ($CD31^+$) LOCs could contribute to endothelial repair and attenuate the neointimal lesion formation in mechanically-injured FAs by their direct incorporation into the arterial endothelium and paracrine effects; however, any EPC treatment could neither rescue the MCT-injured pulmonary vasculature nor attenuate PAH.

2. Materials and methods

2.1. Animals

Wild-type Fisher-344 (F344) rats were purchased from Charles River (Japan). Transgenic rats (F344 background) that ubiquitously express enhanced green fluorescent protein (GFP) were generous gifts from Dr T. Ochiya (National Cancer Center, Tokyo, Japan) [27]. All experimental procedures and protocols were approved by the Institutional Committee for Animal Research of the University of Tokyo and complied with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health [NIH] publication No. 85–23; revised 1996).

2.2. BM-derived EPC cultures and classification

BM cells were harvested from the femurs of 8-week-old male F344 rats. BM-derived mononuclear cells (MNCs) were isolated by density-gradient centrifugation with Pancall 1.099 (Biotech) and resuspended in endothelial cell culture medium (EGM-2; Lonza) supplemented with 1 μg/mL hydrocortisone, 3 μg/mL bovine pituitary extract, 10 ng/mL human recombinant VEGF (R&D Systems), and 10% fetal bovine serum (FBS) (complete EGM-2 medium). As previously described [28], isolated MNCs were cultured at a density of 1.0×10^7 cells per well of a fibronectin-coated 6-well plate (BD Biosciences) at 37 °C, 5% CO_2, in a humidified incubator. After 24 h, non-adherent cells were removed, and the medium was replaced into fresh complete EGM-2 medium. The medium was changed daily for 7 days and then every other day. After 7 days, colonies that originated from adherent cells emerged with cobblestone appearance. We classified BM-derived EPCs into the three subpopulations based on the timings of emergence and the culture periods as follows: early EPCs were defined as attached cells on days 3–6; and LOCs and VLOCs were defined as cells forming cobblestone colonies on days 9–14 and 17–21, respectively. The numbers of emerged colonies and cells comprised in each colony were counted by visual inspection using an inverted microscope every day.

As a rat endothelial cell control, rat aortic endothelial cells (RAoECs) (Cell applications, CAR304K05a) were maintained on the same condition and medium (EGM-2). Rat fibroblasts (Cell systems, ACBRI5118) were maintained in DMEM containing 10% FBS, 2 mM L-glutamine, and 1 mM sodium pyruvate.

2.3. Immunocytochemistry

To identify the cultured cells as EPCs with the classical method [1,28], adherent cells were incubated with 10 μg/mL acetylated low density lipoprotein labeled with 1,1′-dioctadecyl-3,3, 3′,3′-tetramethylindocarbocyanine perchlorate (DiI-Ac-LDL; Molecular Probes) for 4 h at 37 °C. Then, cells were fixed with 2% paraformaldehyde and stained with fluorescein (FITC)-labeled Ulex europaeus agglutinin I (UEA-I) lectin (Sigma) at 10 μg/mL. The cells that are double positive for DiI-Ac-LDL and FITC UEA-I have been classically considered as the prototype of EPCs [1,28]. Separately, the three EPC subpopulations were subcultured in 4-well chamber slides (BD Biosciences), respectively, and fixed in 4% paraformaldehyde for 10 min for immunocytochemistry. The following anti-rat primary antibodies were used for the immunostaining: anti-CD31 and anti-CD68 (AbD Serotec); anti-CD45 (BD Biosciences); anti-CD34, anti-Flk-1 and anti-VE cadherin (Santa Cruz Biotechnology); and anti-vimentin (Millipore). Alexa Fluor 488-conjugated or 555-conjugated secondary antibodies specific to the appropriate species were used (Invitrogen). The nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma). Images were captured using a confocal microscope (Fluoview FV300; Olympus).

2.4. Fluorescence cytometry analysis

Adherent cells of the three EPC subpopulations and RAoECs were trypsinized and analyzed for expression of CD31, Flk-1, CD45, CD34 and CD90. The mouse monoclonal fluorochrome-conjugated anti-rat antibodies were used as follows: anti-CD 31-phycoerythrin, anti-CD45-FITC and anti-CD90-FITC (BD Biosciences); and anti-Flk-1-FITC (Novus). A mouse monoclonal anti-rat CD34 unconjugated antibody (Sigma) was used, followed by the incubation with an anti-mouse secondary antibody conjugated with Alexa Fluor 488 (Invitrogen). Cells without the incubation with primary antibodies were used as negative control. Quantitative fluorescence analysis was performed using a Guava EasyCyte Plus System (Millipore). Data were evaluated with Guava Express Pro software (Millipore). All assays were performed on six independent cultures.

2.5. EPC proliferation assay

Cell proliferative ability was measured using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega), a colorimetric determination of viable cells based on the biological reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS), metabolized in active cells. Each of the