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Therapeutic effects of late outgrowth endothelial progenitor cells or mesenchymal stem cells derived from human umbilical cord blood on infarct repair

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ABSTRACT

Background: This study sought to systematically investigate the derivation of late outgrowth endothelial progenitor cells (late EPC) and mesenchymal stem cells (MSC) from umbilical cord blood (UCB) and to examine their therapeutic effects on myocardial infarction (MI).
Methods: The expression of angiogenic genes was determined by qRT-PCR. Myocardial infarction (MI) was induced in rats, and cells were directly transplanted into the border regions of ischemic heart tissue.
Results: Culture of UCB mononuclear cells yielded two distinct types of cells by morphology after 2 weeks in the same culture conditions. These cells were identified as late EPC and MSC, and each was intramyocardially injected into rat hearts after induction of MI. Echocardiography and histologic analyses demonstrated that both EPC and MSC improved cardiac function and enhanced vascularization, although fibrosis was reduced only in the EPC transplanted hearts. Different paracrine factors were enriched in EPC and MSC. However, once injected into the hearts, they induced similar types of paracrine factors in the heart. Transplanted EPC or MSC were mostly localized at the perivascular areas. This study demonstrated that EPC and MSC can be simultaneously derived from UCB under the same initial culture conditions, and that common paracrine factors are involved in the repair of MI.
Conclusion: Late EPC and MSC are effective for infarct repair, apparently mediated through common humoral mechanisms.

1. Introduction

During the past decade, adult bone marrow (BM) was the major source of stem or progenitor cells such as hematopoietic stem cells (HSC), endothelial progenitor cells (EPC), and mesenchymal stem cells (MSC) for cell-based therapy. However, the aspiration of BM is an invasive procedure associated with risks such as infection, bleeding, or pain. Compared to BM, UCB has various benefits for clinical application due to the lack of viral contamination and easy access without invasive procedures or long-term storage [1]. Therefore, UCB has emerged as an attractive and alternative source for stem or progenitor cells for clinical application [2–4].

To date, at least two distinctive types of cultured EPC have been reported: early and late outgrowth EPC. Early EPC are typically derived from culture of mononuclear cells (MNC) for less than a week and are characterized by a low proliferation rate [5–8]. Late outgrowth EPC, also called outgrowth endothelial cells, late EPC, or endothelial colony forming cells, emerge as colonies after culture of MNC for 2 weeks or more and are characterized by cobblestone morphology and high proliferative potential [9–11]. These two types of EPC express different sets of cell surface markers. Early EPC express the pan-leukocyte marker CD45 and monocytic/macrophage markers CD11b/CD14. Late EPC express most of the endothelial cell markers but not hematopoietic cell markers. MSC are another representative adult stem cell which are frequently

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derived from BM and possess multipotency and paracrine effects [12,13]. Over the past decade, BM-derived early EPC or MSC were shown to improve cardiovascular ischemia or infarct repair [14–19]. However, most studies with EPC or MSC derived from UCB remain at the level of in vitro cell characterization [20–26]. While several studies reported therapeutic effects of UCB-derived early EPC on myocardial infarction (MI), no studies have investigated the therapeutic potential of UCB-derived late EPC or MSC. Only a few studies are available which explored in vitro cardiomyogenic differentiation potential of UCB-derived MSC [27].

Accordingly, the present study sought to characterize UCB-derived late EPCs and MSCs and determine their therapeutic effects in myocardial infarction and the underlying mechanisms.

2. Materials and methods

2.1. Derivation and characterization of EPC and MSC

UCB was collected after receiving informed consent from the birthing mother using the guidelines approved by institutional review boards of Yonsei University. MNC were collected according to the previously published method [28]. Briefly, MNC were obtained by Ficoll-Hypaque (Amersham Biosciences, Sunnyvale, CA) density gradient centrifugation. The MNC were harvested from the interface, washed two to three times, plated on cell culture dishes coated with 0.01% human fibronectin (Sigma, St. Louis, MO) at a density of 0.5 to 1×10^6 cells/cm^2, and cultured in endothelial growth medium-2 (EGM-2, Cambrex, Walkersville, MD). Non-adherent cells were removed during medium changes after 72 h and the adherent cells were fed twice a week with fresh culture medium. Cell expansion culture was performed with EGM-2 for another three months.

2.2. Flow cytometry

Flow cytometry analyses of EPC and MSC were performed at passage 5 of culture as we previously described [29]. For flow cytometry analyses, we used FITC- or PE-conjugated Abs against CD29, CD31, CD34, CD44, CD45, CD73, CD90, CD105, CD144, CD146, vWF, HLA-DR (all from BD), KDR and Tie-2 (from R&D systems). The fluorescence intensities of the cells were evaluated by an FC 500 flow cytometer (BD) and the data were analyzed with FlowJo 7.

2.3. Tube formation assay

To investigate the tube formation potential, EPC and MSC were seeded onto Matrigel-coated dishes (BD) and cultured in complete EGM-2 medium at a concentration of 1×10^4 cells/well. After 24 h of incubation, endothelial network formation was examined and representative fields were photographed under fluorescence microscopy.

2.4. Induction of myocardial infarction and cell transplantation

Male Sprague–Dawley rats (LabAnimal, Korea) and nude rats (Charles River Laboratories, Wilmington, MA) aged 8 weeks were used. All animal protocols were approved by the Institutional Animal Care and Use Committees at the Yonsei University and Emory University. For physiologic and functional studies we used Sprague–Dawley rats and for mechanistic studies we switched to nude rats to better elucidate mechanisms. Rats were anesthetized with Zoletil 50 (100 mg/kg, Virbac), then intubated and ventilated using a small animal ventilation apparatus (Harvard apparatus, MA). MI was induced by ligation of the left anterior descending coronary artery as described previously [28]. 1 h after MI, the rats received 5×10^6 EPC or MSC suspended in 50 μl of PBS intramyocardially. The same volume of PBS without cells was injected as a control. The cells or PBS were injected with a 31-gage needle attached to an insulin syringe.

2.5. Echocardiography

Echocardiography was performed 4 weeks after cell transplantation. The rats were anesthetized with inhalation of isoflurane. The echocardiographic images were obtained using Vivid 7 echocardiographic machine with 12 MHz phased array transducer (GE Medical Systems, Milwaukee, WI) [30]. Left ventricular (LV) M-mode and two dimensional imaging at the papillary muscle level were obtained by parasternal short axis view. The end-systolic inter-ventricular septum (IVSs), end-diastolic inter ventricular septum (IVSd), LV end-systolic dimension (LVESD), LV end-systolic posterior wall (LVPWs), and LV end-diastolic posterior wall (LVPWd) were measured on M-mode echocardiograms. These parameters allowed calculation of the LV fractional shortening (FS %) using the following equation: FS % = [(LVEDD − LVESD) / LVEDD] × 100%. The LV ejection fraction (EF %) was measured by a modified Quinone's method. All measurements were averaged for three consecutive cardiac cycles and all the parameters were analyzed by an observer blinded to the experiment.

2.6. Histological analysis

Rats were euthanized 2 or 4 weeks after cell transplantation. Hearts were harvested and fixed in 4% paraformaldehyde and incubated overnight in 15% sucrose solution. The tissues were embedded in OCT compound (Sakura Finetek), snap-frozen in liquid nitrogen, and sectioned at 10–20 μm thickness as described previously by our laboratory [28]. For capillary density measurement, four frozen sections of ischemic tissues were stained with primary biotinylated isolectin B4 (ILB4) (1:250, Vector Laboratories, Inc., Burlingame, CA) and secondary streptavidin Alexafluor 488 (1:400, Invitrogen). Five fields from four tissue sections were randomly selected, and the number of capillaries was counted in each field. Photographs were taken using fluorescent inverted microscopy or confocal microscopy. To evaluate apoptosis, TdT-mediated dUTP nick-end labeling (TUNEL) reaction was performed using a fluorescein in situ cell death detection kit (Roche-Molecular). To investigate proliferative cells, immunostaining with anti-Ki-67 antibody (Novocastra Laboratories) was performed. Details of the above procedures were described in our prior publications [28,31].

2.7. Real-time RT-PCR (qRT-PCR)

qRT-PCR assay was performed as described previously [32]. Peri-infarct myocardial tissues were harvested and pulverized to extract RNA or protein. Total RNA was extracted using RNA-Stat (Iso-Tex Diagnostics) according to the manufacturer's instructions. cDNA was synthesized from the extracted RNA (500 ng) with TaqMan Reverse Transcription Reagents (Applied Biosystems) and subjected to real-time polymerase chain reaction using human and rat-specific primers (Supplemental Table 1). qPCR was performed on a 7500 Fast Real-Time PCR system (Applied Biosystems) using gene expression master mix and the TaqMan method (Applied Biosystems). Relative mRNA expression of target gene normalized to GAPDH was calculated as previously described [29]. Relative RNA expression was determined using the formula Rel Exp $= 2^{-\Delta CT}$ (fold difference), where ΔCt = (Ct of target genes) − (Ct of endogenous control gene, GAPDH) in experimental samples [29,32].

2.8. Statistical analysis

All data were presented as mean ± SD. Statistical analyses were performed with Student's *t*-test for comparisons between two groups, and ANOVA followed by Bonferroni's correction for more than two groups using SPSS version 11.0. $P < 0.05$ is considered statistical significance.

3. Results

3.1. Culture derivations of late EPCs and MSCs

After culturing UCB-derived MNC for 14–21 days under conventional EPC culture conditions, two types of dominant cell populations emerged in different dishes: cobblestone-like cells (identified as EPC) and spindle-shaped cells (identified as MSC) (Fig. 1A). Due to unknown reasons, one type of cell usually dominates in one dish within several weeks. Cells of each type were further cultured and the distinction became clearer. Spindle-shaped cells or MSC were continuously cultured without significant signs of senescence or morphologic changes for more than 21 passages (over 3 months) and were expanded more than 1000-fold. EPC continued to proliferate for 4 weeks, albeit showing less proliferative potential. The mean doubling time of EPC and MSC were approximately 34 and 26 h, respectively (Fig. 1B). Twenty six units out of 48 units of cord blood (66%) generated EPC and 13 units out of 37 units (35%) generated MSC during cultivation.

3.2. Characterization of late EPC and MSC

Flow cytometry was used to characterize the two cell types. Cobblestone cells expressed CD31 (PECAM-1), CD34, CD105 (Endoglin), CD144 (VE-Cadherin), CD146 (MCAM), KDR (VEGFR-2), TIE-2, HLA-ABC, and HLA-DR, and were negative for CD45 (leukocyte common antigen), suggesting a late EPC phenotype (Fig. 1C). The spindle-shaped cells expressed CD29 (Integrin-β1), CD44 (hyaluronate receptor), CD73 (NT5E), CD90 (Thy-1), and HLA-ABC, but not CD31, CD45 or HLA-DR, showing a conventional MSC phenotype. To investigate the in vitro vessel-forming capabilities, we conducted tube formation assays on Matrigel. The results showed that both EPC and MSC formed tubular structures and EPC showed more branching