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Original article

Detection and characterization of circulating endothelial progenitor cells in normal rat blood

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ABSTRACT

Introduction: There are currently few widely accepted noninvasive detection methods for drug-induced vascular damage. Circulating endothelial progenitor cell (EPC) enumeration in humans has recently gained attention as a potential biomarker of vascular injury/endothelial damage/dysfunction. The rat is commonly used in preclinical drug development toxicity testing and lacks consensus noninvasive methodologies for immunophenotypic identification of EPCs. Identification of immunophenotypic markers of EPCs in the rat would enable transfer of technologies use`d in human for potential development of biomarkers for vascular injury the rat. Therefore, the aim of this work was to develop methods to consistently identify a discreet population of EPCs from rat peripheral blood. **Methods:** EPCs were identified phenotypically from rat blood using cell culture, immunolabeling, fluorescence microscopy, and flow cytometry. EPCs isolated using immunolabeling coupled with magnetic separation and flow cytometric cell sorting were characterized genotypically using mRNA analysis. **Results:** A modified colony forming unit (CFU)-Hill assay confirmed existence of immature EPCs in peripheral blood. Extended in vitro culture resulted in a morphology and immunophenotype consistent with mature endothelial cells as noted by positive staining for CD31, von Willebrand factor, rat endothelial cell antigen, and negative staining for smooth muscle cell α-actin. The majority of the cells identified as LDL^{+}/CD11b/c^{-} did not stain positively for either vWF or CD31. EPC populations isolated using magnetic separation and cell sorting were consistently positive for *PECAM1*, *EDN1*, *FLK1*, *VWF*, *ITGAD*, *CCR1*, *IP30*, and *MMP2* mRNA expression. Cells identified as EPCs express cell-surface and gene expression markers consistent with endothelial cells and endothelial progenitor cell populations. **Discussion:** Vascular trauma induces transient mobilization of EPCs in humans and their enumeration and characterization have been proposed as a surrogate biomarker for assessment of vascular injury. Potential exists for using rat circulating EPCs as a surrogate sampling population for biomarker development in drug-related injury in preclinical toxicity studies. A prerequisite to biomarker development is the ability to consistently identify a discreet population of EPCs from peripheral rat blood. This work describes novel methods for isolation and validation of phenotypically and genotypically consistent populations of rat EPCs from peripheral blood. These methods are well suited for potential future use in validation of enumeration and/or biomarker development methods in the rat.

1. Introduction

Acute vascular injury has been correlated with an increase in the number of circulating endothelial cells (CECs) and bone marrow derived endothelial progenitor cells (EPCs) in the peripheral blood (Wu, Chen, & Hu, 2007; Gill et al., 2001). Under normal circumstances EPCs account for approximately 0.1% of peripheral blood, have proliferative potential, and can differentiate into mature endothelial cells. When required for vascular repair/angiogenesis or in cases of vascular stress, EPCs enter the peripheral blood and migrate to areas of endothelial damage to begin the reparative process (Yao et al., 2008). EPCs then can differentiate into damaged endothelium (Real, Caiado, & Dias, 2008).

Vascular trauma induces a very rapid but transient mobilization of a significant number of EPCs in humans (Gill et al., 2001). Quantitation of circulating endothelial cells and EPCs has been proposed as a surrogate biomarker for assessment of vascular injury in humans (Wu et al., 2007). Acquiring consensus methods for identification and enumeration of circulating EPCs in toxicology studies may enable transfer of technologies used in human to provide a noninvasive surrogate biomarker for assessment of vascular injury in rats. Although the rat is commonly used in preclinical drug development

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efficacy and toxicity testing, there is little published data currently available describing flow cytometric identification and quantitation of circulating EPCs and culture of EPCs isolated from rat blood and these have been inconsistent or contradictory (Yao et al., 2008; Real et al., 2008).

The objectives of this study were to develop methods to confirm the presence of a discreet population of circulating EPC in normal rat blood using cell culture and fluorescence microscopy, identify EPC by flow cytometry, and confirm characteristics of both endothelial and progenitor genotype using TaqMan® mRNA expression analysis. This work describes novel methods for peripheral blood sampling and validation of phenotypically and genotypically consistent populations of rat EPCs. These methods are well suited for potential future use in validation of enumeration and/or biomarker development methods in the rat. Disparate methods have been described for culture and identification of putative EPC colonies (Real et al., 2008; Leor and Marber, 2006). Since a reagent kit for colony forming unit (CFU)-Hill assay is commercially available, this method was chosen for the current study. This kit was designed for use with human blood and required a few modifications to permit growth of cells derived from rat blood. The resulting modified CFU-Hill assay confirmed the existence of immature EPCs in peripheral blood. Extended in vitro culture resulted in a morphology and immunophenotype consistent with mature endothelial cells as noted by positive staining for CD31, von Willebrand factor, rat endothelial cell antigen, and negative staining for smooth muscle cell α-actin. Putative EPCs from lysed whole rat blood were identified by DiI-AcLDL uptake (Thorne, Mhaida, Ralston, & Burns, 2007; Pluddemann, Neyen, & Gordon, 2007; Voyta, Via, Butterfield, & Zetter, 1984; Martin et al., 2007). These cells are referred to as LDL^+ in this manuscript. An anti-CD11b/c antibody was used to exclude monocytes/macrophages that may have taken up DiI-AcLDL, as well as granulocytes, from analysis (Draude, von Hundelshausen, Frankenberger, Ziegler-Heitbrock, & Weber, 1999). DiI-AcLDL uptake occurs via the "scavenger cell pathway" (Voyta et al., 1984) and accordingly, labeling of CD36 scavenger receptor was also used to identify and isolate putative EPCs for further examination; these cells will be referred as $CD36^+$ cells.

TaqMan® real-time PCR analysis was performed on LDL^+ and $CD36^+$ cell populations using a panel of genes (Table 1) regarded to be constitutively expressed in and critically related to function of endothelial cells (Fish and Marsden, 2006; Smirnov et al., 2006). $CD36^+$ cells demonstrated consistent, uniform expression of FLK1, PECAM, END1, and vWF; mRNA isolated from LDL^+ cells contained transcripts for PECAM, END1, and vWF. Each of the four genes examined (Table 2) which are purported to be more highly expressed in EPC (Fuharata et al., 2007) than mature cultured endothelial cells. *ITGAD*, *IP30*, *CCR1*, and *MMP2* were consistently and uniformly expressed in $CD36^+$ cells and expression of each was high compared with their expression in mature rat heart. These data indicate that EPCs, identified as described here, express cell-surface and gene expression markers consistent with endothelial and progenitor cell populations. This work details novel methods for the isolation and validation of rat EPCs from peripheral blood. The results of the genotypic analysis of these cells confirm that the cell population extractable by these methods from rat blood consists of circulating endothelial progenitor cells that can be successfully isolated and purified for further analysis. This non-invasive approach provides the opportunity to assay a readily available endothelial source in order to investigate toxicologic or pharmacologic effects on endothelial cells after treatment with pharmaceutical agents.

Table 1
Endothelial cell genes investigated by TaqMan® real-time PCR.

Protein name(s)	Gene	Gene aliases
Tek, endothelial-specific receptor tyrosine kinase; CD202B	*TEK*	*TIE2, VMCM TIE-2,*
Melanoma cell adhesion molecule; CD146	*MCAM*	*CD146, MUC18*
Endothelin 1	*EDN1*	*ET1, ET-1*
Vascular endothelial growth factor	*VEGF*	*MGC70609*
von Willebrand factor; factor VIII-related antigen	*VWF*	*VWD, F8VWF*
Vascular endothelial growth factor receptor 1	*FLY1*	*FLT-1, VEGFR1*
Kinase insert domain receptor; endothelial growth factor receptor 2	*FLK1*	*KDR. FLK-1, VEGFR2*
Platelet/endothelial cell adhesion molecule; CD31	*PECAM1*	*PECAM-1, CD31*
Cadherin 5, type 2; CD144; VE-cadherin (vascular endothelium)	*CDH5*	*CD144, 7B4*

Table 2
Progenitor cell genes investigated by TaqMan® real-time PCR.

Protein name(s)	Gene	Gene aliases
Integrin, alpha D; integrin, alpha X, CD11c; leukocyte adhesion glycoprotein	*ITGAD*	*ITGAX*
Chemokine receptor 1; macrophage inflammatory protein 1 alpha receptor; RANTES receptor	*CCR1*	*HM145, MIP1aR, RANTES-R*
Interferon gamma inducible protein 30	*IP30*	*IF130*
Matrix metallopeptidase 2; gelatinase A; 72 KDa type IV collagenase	*MMP2*	

2. Materials and methods

2.1. Materials

3% acetic acid/methylene blue and EndoCult® Liquid Medium Kit were purchased from Stem Cell Technologies, Vancouver, BC, Canada. Histopaque® 1083, bovine serum albumin (BSA) and Pronectin-F were purchased from Sigma-Aldrich, St. Louis, MO. BD BioCoat fibronectin-coated 6 and 24-well plates were purchased from BD, Bedford, MA. Mouse anti-human smooth muscle actin-α and rabbit anti-human vWF were purchased from Dako, Carpinteria, CA. Mouse anti-rat cell antigen (RECA)-1 was purchased from Abcam, Cambridge, MA. Alexa Fluor® 488 (A488) goat anti-mouse IgG, Alexa Fluor® 488 goat anti-rabbit IgG, Zenon™ APC mouse IgG Labeling Kit, Aminostilbamidine methanesulfonate (ASBMS), 1,1′-dioctadecyl–3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate-acetylated-low density lipoprotein (DiI-acLDL), and 7-aminoactinomycin D (7AAD), were purchased from Invitrogen, Carlsbad, CA. Fluorescein-conjugated polyclonal sheep anti-rat von Willebrand factor (vWF-FITC) and biotin-conjugated mouse anti-human CD31 (CD31-biotin, clone WM59) were purchased from Serotec, Raleigh, NC. Purified mouse anti-rat CD31 (clone TLD-3A12), R-phycoerythrin-Cy7-conjugated mouse anti-rat CD42d (CD42d-PECy7, clone RPM.4), fluorescein-conjugated mouse anti-rat CD3 (CD3-FITC, clone G4.18), mouse anti-rat CD45R (CD45R-FITC, clone HIS24), mouse anti-rat CD11b/c (CD11b/c-FITC, clone OX-42), mouse anti-rat CD42d (CD42d-FITC, clone RPM.4), purified mouse anti-mouse CD36 scavenger receptor (clone, CRF D-2712), and streptavidin-allophycocyanin-Cy7 (SA-APCCy7) were purchased from BD Pharmingen, San Diego, CA. Custom conjugation of purified CD36 scavenger receptor to R-phycoerythrin (CD36-PE) was performed by ReaMetrix, San Carlos, CA. Mouse anti-rat CD11b/c (clone OX-42) was purchased from Accurate Chemical, Westbury, NY. APC mouse anti-rat CD11b/c (CD11b/c-APC, clone OX-42) was purchased from BioLegend (San Diego, CA). Anti-PE MicroBeads were purchased from Miltenyi Biotec Auburn, CA. RPMI-1640 medium and Hanks' Balanced Salt Solution with Calcium/Magnesium (HBSS) were provided by GSK media prep lab. Complete RPMI consisted of RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100 IU penicillin, and 100 μg/mL streptomycin (pen/strep, Invitrogen).

The Absolutely RNA® Microprep Kit (Stratagene, La Jolla, CA) and the RNA Clean-Up Kit™-5 (Zymo Research Labs, Orange, CA) were used to extract and concentrate RNA from samples, respectively, according to each manufacturer's instructions. Normal rat heart total RNA (Ambion, Austin, TX) was used as positive control for cDNA