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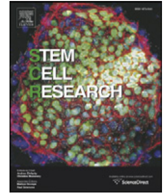

Initial clonogenic potential of human endothelial progenitor cells is predictive of their further properties and establishes a functional hierarchy related to immaturity

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ABSTRACT

Endothelial progenitor cells (EPCs) generate *in vitro* Endothelial Colony Forming Cells (ECFCs) combining features of endothelial and stem/progenitor cells. Their angiogenic properties confer them a therapeutic potential for treating ischemic lesions. They may be isolated from umbilical cord blood (CB-ECFCs) or peripheral adult blood (AB-ECFCs). It is generally accepted that CB-ECFCs are more clonogenic, proliferative and angiogenic than AB-ECFCs. Nevertheless, only a few studies have focused on the functional heterogeneity of CB-ECFCs from different individuals. Moreover, AB-ECFC loss of function is yet to be precisely described. We have focused on these two issues that are critical for clinical perspectives.
The detailed clonogenic profile of CB-ECFCs and AB-ECFCs was obtained and revealed a high inter individual heterogeneity and the absence of correlation with age. Most CB-ECFCs yielded initial colonies and had functional properties similar to those of AB-ECFCs. Conversely, a high clonogenicity was associated with an enhanced proliferative and angiogenic potential and stemness gene overexpression, confirming that immaturity, lost by AB-ECFCs, was a prerequisite to functionality. We thus demonstrated the importance of selecting CB-ECFCs according to specific criteria, and we propose using the initial clonogenicity as a relevant marker of their potential efficacy on vascular repair.
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1. Introduction

Since their discovery by Asahara's team in 1997, endothelial progenitor cells (EPCs) have aroused researcher interest for angiogenic cell therapies to treat ischemia (Asahara, 1997). The term “EPCs” refers to several heterogeneous cell populations with different phenotypes and angiogenic potential. *In vitro*, two main cell types have been identified: early EPCs or Colony Forming Unit-Endothelial Cells (CFU-ECs) with a myeloid phenotype characterized by a paracrine angiogenic effect and late EPCs or Endothelial Colony Forming Cells (ECFCs), also known as Outgrowth Endothelial Cells (OECs) (Yoder et al., 2007) which participate directly in neoangiogenesis.

Umbilical cord blood-derived Endothelial Colony Forming Cells (CB-ECFCs) display an endothelial phenotype ($CD31^+/CD144^+/VEGFR\text{-}2^+/CD45^-/CD14^-$) associated with progenitor cell features such as clonal growth, high proliferation (Bompais et al., 2004; Ingram et al., 2004), stemness gene expression and an enhanced reprogramming efficiency into induced pluripotent stem cells compared to mature endothelial cells (Guillevic et al., 2016). They also show a relative differentiation plasticity since they may acquire specialized endothelial cell features under appropriate external instructive stimuli (Boyer-Di Ponio et al., 2014). Moreover, CB-ECFCs may integrate vessel wall and participate directly in neoangiogenesis in rodent models (Melero-Martin et al., 2007; Au et al., 2008). Besides, several studies have shown their efficacy to enhance revascularization in ischemic brain (Moubarik et al., 2011), limb (Schwarz et al., 2012) and myocardium (Kang et al., 2013). However, some of these features are lost by ECFCs derived from adult peripheral blood (AB-ECFCs). Indeed, they present a decreased clonogenicity, a

Abbreviations: EPCs, endothelial progenitor cells; ECFCs, endothelial colony forming cells; CB, cord blood; AB, adult blood.

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low proliferation and their angiogenic potential is impaired, even when they are obtained from healthy donors (Ingram et al., 2004; Au et al., 2008). These findings raise many questions about the validity of the efficacy of autologous cell therapy and highlight the need to better understand ECFC functional impairment in aging in order to identify targets to prevent this dysfunction. Thus, despite a potential alloantigenicity which may require MHC-matched donors, umbilical cord blood remains described as one of the most valuable sources of functional ECFCs to be adapted for cell therapy. Nevertheless, the clinical use of CB-ECFCs requires optimization and rationalization, especially by selecting the most angiogenic cells.

Since the discovery of these cells (Ingram et al., 2004), secondary colonies have been generated in several studies to assess the immaturity of ECFCs from blood or vascular wall and hierarchize them (Alphonse et al., 2015; Ingram et al., 2005; Patel et al., 2016). The aim of this work was to identify relevant information directly from the initial number of colonies obtained at the first isolation step of CB-ECFCs and AB-ECFCs.

For several years that we grow CB-ECFCs, we have observed a high heterogeneity in colony yields (from 0 to over 100 colonies). In the present study, we investigated whether their initial clonogenicity impacted CB-ECFC properties and could be used as a predictive marker of their functionality. A key issue was also to demonstrate that ECFC stemness was a functional prerequisite which is completely lost by AB-ECFCs.

2. Materials and methods

2.1. ECFC isolation and culture

Human samples were collected and handled in compliance with the declaration of Helsinki. Umbilical cord blood samples from healthy full-term newborns were obtained through a partnership with the Cord Blood Bank of St Louis Hospital (Paris, France) which is authorized by the French Regulatory Authority (authorization no. PPC51) and participates in scientific research. Human peripheral blood from healthy male adults was obtained through a partnership with the French Establishment of Blood (EFS, Ile de France, authorization 14/5/011). Legal age to give blood ranges from 18 to 70 years old. This activity was declared to and authorized by the French Ministry of Research under number AC-2008-376, and to the French Organization for standardization under number 201/51848.1.

Mononuclear cells (MNC), obtained by density gradient centrifugation, were seeded onto rat tail collagen type I (Becton Dickinson BD, Le Pont de Claix, France) -coated wells as previously described (Boyer-Di Ponio et al., 2014).

ECFC colonies appeared after 7–20 days of culture. From passage 1 (P1), cells were seeded at 5000 cells/cm^2 and grew in EGM-2 MV medium (Lonza, Köln, Germany). Population doubling (PD) was determined by the following formula: $\log^2 (n_f/n_o)$, where n_o is the cell number initially seeded and n_f the cell number at confluence obtained at each passage. Cumulative population doubling (CPD) is the sum of all previous PDs. Population Doubling Time (PDT) was calculated using the following formula: t/PD, where t is the culture time interval between each passage.

2.2. BrdU and CFDA SE proliferation assays

Proliferative potential of CB and AB-ECFCs was assessed using BrdU Cell Proliferation Assay Kit #6813 (Cell Signaling) and Vybrant® CFDA SE Cell Tracer Kit (Life technologies) according to the manufacturer's instructions (More details in supplementary material).

2.3. Senescence-associated β-galactosidase activity assay

Cells were seeded in triplicate in 12-well plates at 5000 cells/cm^2. At 80% of confluence, cells were fixed and senescence-associated β-galactosidase activity was revealed using the Senescence Cells Histochemical Staining Kit (Sigma-Aldrich, St-Louis, USA) according to the manufacturer's instructions. Positive (blue) and negative cells were counted manually from 3 phase contrast pictures per well using ImageJ cell counter.

2.4. Western blotting

To reveal p16 protein expression, 30 μg of total protein extract, obtained using CelLytic™ M Cell Lysis reagent (Sigma-Aldrich, St-Louis, USA), were blotted onto nitrocellulose membranes (Bio-Rad, USA). The non-specific binding sites were blocked by incubation in Odyssey Blocking Buffer (Li-Cor biosciences, Lincoln, USA) for 1 h at room temperature (RT) under gentle agitation. The membranes were then cut in two parts and incubated overnight at 4 °C, one with p16 primary rabbit polyclonal antibody (0.4 μg/mL, Santa Cruz Biotechnology) and the other with β-actin primary mouse IgG1 antibody (1 μg/mL, MBL Life Science) diluted in Odyssey Blocking Buffer. After washing, they were incubated with Odyssey's secondary antibody for 45 min at RT and scanned using the Odyssey imaging system.

To reveal p21 and p53 protein expression, 20 μg of total protein extract were blotted onto PVDF membranes (GE Healthcare, Life Sciences, Germany). The non-specific binding sites were blocked by incubation in TBS-Tween 0.2% + BSA 5% for 1 h at room temperature (RT) under gentle agitation. The membranes were then cut in two parts and incubated overnight at 4 °C, one with p21 primary rabbit polyclonal antibody (1 μg/mL, Santa Cruz Biotechnology) and the other with p53 primary mouse monoclonal IgG1 antibody (1 μg/mL, Santa Cruz Biotechnology) diluted in TBS-Tween 0.2% + BSA 5%. After washing, they were incubated with HRP coupled secondary antibody and β-actin directly HRP coupled antibody (1 μg/mL, Santa Cruz Biotechnology) for 45 min at RT. HRP was detected with an Immobilon Western kit (Millipore, Molsheim, France).

2.5. Flow cytometry

Fluorescence-activated flow cytometry was performed using BD Accuri™ C6 flow cytometer (BD) and a minimum of 10,000 events were analyzed for each sample. The cells were harvested, washed in 0.5% Bovine Serum Albumin (BSA) in PBS and incubated for 45 min at 4 °C with primary antibodies to analyze the expression of endothelial cell surface marker proteins: FITC-conjugated mouse anti-human CD31 IgG1 (dilution: 1/50, BD Pharmingen, USA), PE-conjugated mouse anti-human CD144 IgG1 (dilution: 1/10, Beckman Coulter), Alexa Fluor 647-conjugated mouse anti-human CD309 (VEGFR2) IgG1 (dilution: 1/5; BD Pharmingen, USA), FITC-conjugated mouse anti-human CD45 (dilution: 1/50, Beckman Coulter) and PE-conjugated mouse anti-human CD34 IgG2a (dilution: 1/50, Miltenyi Biotec, Germany). All the antibodies were used at concentrations suggested by the suppliers according to the cell number. Antibodies and matched isotype controls were incubated for 30 min at 4 °C. Cell viability was assessed with 7-aminoactinomycin D (dilution: 1/20, BD Pharmingen, USA).

2.6. RNA extraction, reverse transcription and quantitative RT-PCR

Total RNA was extracted using RNeasy mini or micro kit (Qiagen, Courtaboeuf, France). Reverse transcriptions were performed with High Capacity cDNA RT Kit (Applied Biosystems, Fischer Scientific, Illkirch, France) according to the manufacturer's instructions.

Expression of *NOSIII* and the stemness genes *DNMT3B*, *GDF3* and *SOX2* was evaluated by Quantitative Taqman RT-PCRs in triplicate according to the manufacturer's instructions using 7000 Real-Time PCR system (Applied Biosystems). Expression of cell cycle regulator genes was screening with *TaqMan® Array Human Cyclins and Cell Cycle Regulation 96-well Plate* (Applied Biosystems) according to the manufacturer's instructions. The relative transcriptional level of each

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