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Are endothelial outgrowth cells a potential source for future re-vascularization therapy?

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ABSTRACT

Endothelial progenitor cells (EPCs) represent a heterogeneous cell population that is believed to be involved in vasculogenesis after ischemic diseases. EPCs could have a potential for future therapies with the purpose of enhancing endothelial repair. However, due to the low amount of these cells in circulation they have to be expanded in vitro before administration into recipients. The purpose of this study was to analyse and evaluate possible changes in morphology and functionality as a result of in vitro ageing of a subtype of EPCs called endothelial outgrowth cells (EOCs), since such changes might compromise the cells' ability to participate in vasculogenesis.
EOCs were isolated and grown from human umbilical cord blood using two methodologies with varying degree of cell purification. The changes between the two culture setups and the changes occurring in EOCs over time were traced by flow cytometry and assays for growth, tube formation, and beta-galactosidase production.
The cells showed to be indistinguishable from each other during the first weeks of culture. The cells showed a changed morphology with bigger and more granular cells and the growth rate decreased with time. The cells also showed an increased Beta-galactosidase expression and decreased tube formation ability in late passage EOCs.
Our data indicates that EOCs undergo senescence during long-term expansion and therefore time for cell harvest has to be validated in order to achieve functional cells still maintaining a therapeutic potential. A possible application in large animal or humans could be local injection of EOCs into affected areas and thereby reducing the need for long-term expansion of the cells.

1. Introduction

Recent reports on the possible role of endothelial progenitor cells (EPCs) in ongoing repair of blood vessel endothelium and in revascularization in tissue suffering from ischemia (Barclay et al., 2012; Urbich and Dimmeler, 2004) indicate that administration of these cells in the future may have a therapeutic value in different diseases involving impaired blood supply. EPCs can be harvested from different sources (Timmermans et al., 2009) for example peripheral circulating blood, umbilical cord blood or bone marrow samples. However, the number of EPCs that can be obtained varies dependent on the source, as umbilical cord blood contains more than 10-fold excess of cells staining positive for the EPC surface marker CD34 compared with adult human peripheral blood (Murohara et al., 2000). In the present study we focus on EPCs from umbilical cord blood, however in future studies it will be highly relevant to study the effect on organismal ageing on EPC functionality, by characterising EPCs isolated from people of different ages. Studies characterising (Hirschi et al., 2008; Yoder and Ingram, 2009; Yoder et al., 2007) and evaluating the therapeutic potential of EPCs have, nevertheless, generated conflicting results (Kalka et al., 2000; Nolan et al., 2007; Pagan et al., 2013; Purhonen et al., 2008). Endothelial progenitor cells seem to be a heterogeneous population of cells which can be divided into several subpopulations dependent on origin and function (Ingram et al., 2005; Timmermans et al., 2009). The early EPCs (eEPCs) and the EOCs are the main types isolated in vitro (Hur et al., 2004; Medina et al., 2010; Timmermans et al., 2007). In terms of morphology and growth, eEPCs appear after 1 week as spindle-shaped cells with low proliferative potential and EOCs appear after 4 weeks as cobblestone-shaped cells with clonogenic and high proliferative potential (Ingram et al., 2004; Reinisch et al., 2009; Yoder et al., 2007).

Due to relatively few EPCs found in the blood, a multiplication in culture is necessary even if used in small animal models. Little is though

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known about possible functional and morphological changes appearing during long-term expansion in culture. Recent research has shown that EOC senescence limited the cells regenerative potential by impairing vasoreparative properties in vitro and in vivo by mechanisms involving cytokines like IL-8 (Medina et al., 2013). Therefore, late passage EOCs might be preferable in studies of ageing processes in the blood vessels such as reduced endothelial repairment and angiogenesis capacity (Izzo and Mitchell, 2007; Sadoun and Reed, 2003). In contrast, early passage EOCs might be suited for studying any reparative capacity of these cells in angiogenesis or vasculogenesis.

In the present study we provided phenotypic and functional analysis of EOCs from culture start to onset of senescence, and show that the changes occurring to EOCs over long-term cell culturing alter their tubulogenic potential.

2. Materials and methods

2.1. Cell culture

Human umbilical cord blood samples (20–90 ml) from healthy newborns were collected and mononuclear cells (MNC) were isolated by density gradient centrifugation. Briefly, blood was diluted 1:3 in phosphate-buffered saline (PBS) and layered over Ficoll paque plus. MNCs were washed and divided into two groups handled in two diverse ways. In the first group MNCs were immediately transferred to wells; in the other MNCs were put on a column for purification into a CD34 cell fraction and then put in culture. Cells were seeded at a density of $1–1.8 \times 10^6$ cells/2 cm^2 on gelatine (Sigma-Aldrich) and maintained in endothelial basal medium-2 (EBM-2) supplemented with EGM-2 mv Single-Quots (EGM-2 mv medium, Lonza) and were incubated (5% CO_2, 37 °C) for 3–4 weeks until 80% colony confluence. Medium was changed every two days for the first seven days and then twice a week during the following passages. The cells were propagated for 60 days in culture and cell numbers were determined using trypan blue exclusion test. Population doublings (PD) were estimated using the following equation:

$$PD = \frac{\log 10(\textit{cells harvested at the end of passage}) - \log 10(\textit{cells seeded at the beginng of passage})}{\log_{10} 2}.$$

For each passaging the resulting PD estimation was added to the sum of PD from the previous passages to achieve the cumulative population doubling level (CPDL), which was plotted against days in culture. It should be noted that the number of cells obtained at the first passage were taken as the starting point for CPDL calculation. Early passage EOCs were, in this study, defined as cells that were in their exponential phase of the growth curve whereas late passage cells were cells that were near their Hayflick limit (Hayflick and Moorhead, 1961).

2.2. Flow cytometry

Cells were resuspended in PBS and incubated with respective antibodies for 30 min at room temperature. The following antibodies were used; Abcam: CD146, vWF, CD31, CD14; Miltenyi Biotec: CD34, CD133; BD Bioscience: CD45; R&D: Vascular endothelial growth factor 2 (VEGFR2); and eBioscience: FcERI, CD69, CD105, CD163, CD144. After staining, cells were washed and analysed using flow cytometry (FACSCanto™II, BD Biosciences) and Flow Jo (version 9.3.1, Tree Star Inc.). At least 10,000 events were acquired for each sample and dead cells were excluded using gates defined upon staining with 7-amino-actinomycin D (7-AAD; BD Biosciences).

2.3. Tube formation assay

Cell culture plates were coated with BD Matrix Growth Factor Reduced Matrigel™ (BD Biosciences, San Jose, CA) at 4 °C. The gel was allowed to polymerize in the 37 °C CO_2-incubator for at least 30 min before seeding of 20,000 cells on top of the gel. The cells were allowed to attach and differentiate overnight. Tubes were visually examined with phase contrast using either 2.5× or 10× objective and pictures were recorded digitally with a Zeiss Axiocam MRc camera (Carl Zeiss Micro Imaging) attached to the microscope.

2.4. Senescence—SA-β-galactosidase activity

Cells were examined for senescence associated β-galactosidase activity (SA-β-gal) (Dimri et al., 1995). Briefly, cells were washed twice with PBS and fixed with 2% formaldehyde, 0.2% glutaraldehyde solution in PBS for 3–5 min at room temperature. The fixed cells were washed twice with PBS before addition of 1 mg/ml X-gal in NN-dimethylformamide, 2 mM $MgCl_2$, 150 mM NaCl, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 40 mM citric acid/Na phosphate buffer, pH 6.0. Cells were incubated with the staining solution overnight at 37 °C and washed twice with H_2O and allowed to dry before visual examination in an inverted light microscope.

2.5. Statistical analysis

Results are presented as mean ± 1 SE. A one way ANOVA was used to determine statistical significance of difference between groups (Sigma plot version 12). $P < 0.05$ was considered to indicate a significant difference.

3. Results

3.1. Cell cultures

Human EOCs were grown from two different cell culture groups, respective from MNCs (Fig. 1A) or from CD34 purified cells (Fig. 1B). Immunophenotypic analysis was performed by flow cytometry with the relevant surface markers to show the difference in cell populations between the two cell groups at culture onset (Fig. 1C). The proportion of vWF, CD31, CD105, CD133, CD45, as well as CD34 was higher in the cell group with CD34 purified cells compared to the group with MNCs, however, as expected the expression of CD163, CD144, CD146, VEGFR2 and CD14 was relatively low in both culture groups (Fig. 1C).

The two different cell culture groups were grown until onset of senescence, which was at approximately 60 days. Both groups of produced EOCs had a growth curve that showed a highly proliferative potential for the first 45 days, where after growth ablated (Fig. 2A) and cells reached the onset of Hayflick limit. As the morphology pictures show (Fig. 2A), both early passage EOCs and late passage EOCs exhibited the characteristic cobblestone morphology described for endothelial cells, with the majority of cells being rather small and slightly spindle shaped (Fig. 2A). However, when cells were allowed to age in culture, they became larger in size and more granular for both cell groups as shown in FS–SS diagrams (Fig. 2B). The cultures consisted of one main population of cells having nearly the same size and granularity and only a small subpopulation of EOCs with different size and granularity (Fig. 2B). The DNA dye 7-AAD was used to distinguish viable and apoptotic/dead cells in the cultures. There was no significant difference between the two groups of EOCs in both early and late passage EOCs and comparing early passage EOCs to late passage EOCs a minor raise in percentage of cells incorporating 7-AAD was seen (Fig. 2C). We analysed the cell surface marker expressing profile of early and late passage EOCs from both cell groups after excluding dead cells using 7-AAD. Comparing early passage EOCs and late passage EOCs, both EOC groups were composed of cells with a high expression of CD31 and vWF. Additionally, they had expression of endothelial marker VEGFR2 and the progenitor cell marker CD34 (Fig. 2D). In early passages a small fraction of cells did also express the CD133 marker, though this expression diminished within the first cell passages (Fig. 2D). Moreover, the cells