Endothelial progenitor cell culture and differentiation in vitro: a methodological comparison using human umbilical cord blood

Juliane Eggermann, Stefanie Kliche, Gergely Jarmy, Karin Hoffmann, Ulrike Mayr-Beyrle, Klaus-Michael Debatin, Johannes Waltenberger, Christian Beltinger

Abstract

Objective: Endothelial progenitor cells (EPC) can contribute to vascular repair and targeted tumour therapy. Little is known about generating EPC from human umbilical cord blood. We therefore compared methods for purification of EPC from human umbilical cord blood.

Methods: Mononuclear cells were isolated from human umbilical cord blood by density gradient centrifugation and used either unselected or after CD34 preselection. Unselected mononuclear cells were cultured for 9 days. Culture-dish-adherent (CDAC) and non-adherent (CDNAC) CD34+ cells were cultured separately for 4 weeks. Surface markers were assessed by immunofluorescence staining and FACS analysis.

Results: In unselected mononuclear cells, VEGF-R2 and VE-cadherin expression increased up to day 6. They stained positive with UEA-1 and took up acetylated LDL. Expression of CD45 and CD14 decreased over time, but remained strong. CD133 and CD34 were not expressed. CD34+CDNAC acquired an endothelial phenotype over time with an increase of VEGFR-2 and von Willebrand factor (vWF). CD45 and CD14 decreased, while CD34 and the progenitor-cell marker CD133 remained strongly expressed. CD34−CDAC showed a strong increase in VEGFR-2, CD133, CD34 and vWF, while CD14 decreased, and CD45 did not change.

Conclusion: Putative EPC can be obtained from human umbilical cord blood. When selected for CD34, cells can be differentiated in culture to express markers of mature endothelial cells, while keeping progenitor markers. In contrast, short-term culture of unselected mononuclear cells leads to an endothelioid–monocytoid phenotype devoid of progenitor markers. Thus, the outgrowth from CD34-selected cells appears to be superior to short-term culture of unselected mononuclear cells with regard to endothelial cell-lineage specific differentiation of cells with a progenitor marker profile.

Keywords: Endothelium; Progenitor cells; Adult stem cells; Surface markers; Cell therapy

1. Introduction

Endothelial progenitor cells from bone marrow or peripheral blood play an important role in various physiological and pathophysiological processes. They participate in angiogenesis and arteriogenesis [1], thus being functionally important in vascular repair. Animal studies revealed that neovascularisation of ischemic tissue can be enhanced by autologous bone marrow transplantation [2]. Precursor cells are also known to play a critical role in tumour angiogenesis [3].
Promising therapeutic strategies are based on the concept of endothelial progenitor cells (EPC) being differentiated into mature endothelial cells (EC). These cells may contribute to vascular repair processes and are expected to be of use for targeted antiangiogenic therapy of malignant tumours [3]. For future therapeutic use of putative endothelial progenitor cells further insight into their differentiation, marker profile and potential physiological role is needed.

So far, no uniform definition of an ‘endothelial progenitor cell’ has been proposed. In previous studies putative EPC were obtained from various cellular sources and definition of EPC varies considerably in the literature. Early reports described the isolation and culture of EPC from circulating mononuclear cells. These cells showed morphological signs of endothelial cells and expressed endothelial markers starting between days 3 and 7 of culture [4]. This simple and feasible way to generate putative EPC has been pursued by other groups. Thus, Dimmelé et al. showed that short-term culture of mononuclear cells over 4–7 days leads to adherence and differentiation of putative EPC as defined by the expression of VEGF-receptor-2 (VEGFR-2), vWF, VE-cadherin and CD31 [5]. Recently, endothelial progenitors have been described in short-term cultures (7 days) of mononuclear cells derived from HUCB [6]. These cells were positive for the endothelial lineage markers VEGFR-2, VE-cadherin and CD31, stained positive for endothelial constitutive NO synthase, took up acetylated LDL and expressed CD34. Most cells did not express the hematopoietic lineage marker CD45.

Lin et al. described endothelial outgrowth from freshly drawn peripheral blood anduffy coat [2]. These late outgrowth cells were positive for endothelial markers such as P1H12, thrombomodulin, VEGFR-2, VE-cadherin and CD31 and did not express CD14. Of note, the cells could be expanded exponentially for a prolonged period of time. Other authors favoured a definition of EPC by the newly discovered surface marker CD133 [7,8], expression of which is limited to progenitor cells. Besides CD133, one of the earliest markers expressed on precursor cells is VEGFR-2 [9]. CD34 is another so-called stem cell marker expressed at a very early developmental stage. The group of Rafii cultured cells from bone marrow, fetal liver or peripheral blood from G-CSF-stimulated patients for 4 days in order to allow cells from the monocyte–macrophage lineage to adhere to the culture dish [10]. The cells of interest—putative EPC—then remained within the supernatant. They were removed and cultured separately. In order to limit the unspecific cellular fraction within the culture, mononuclear cells were first preselected for CD34. EPC were defined as VEGFR2⁺/AC133⁺/CD34⁺ cells.

Quirici et al. reported endothelial differentiation of precursor cells from bone marrow using CD133 selection [11]. After reselection with UEA-1 these cells had a high proliferation rate and expressed endothelial but not hematopoietic or progenitor markers. Gehling et al. used CD133-positive progenitor cells from mobilised peripheral blood and differentiated them into adherent endothelial progenitors and non-adherent cells with both hematopoietic and endothelial character [12]. Although human umbilical cord blood (HUCB) has been previously shown to be a potentially useful source to obtain putative EPC [6], little is known about culture conditions and differentiation of precursor cells from HUCB. The therapeutic usefulness of EPC derived from HUCB will most likely be limited due to immunological incompatibilities. However, purification of EPC from peripheral blood of individual patients is difficult due to the very low number of circulating EPC. Thus, generation of EPC from HUCB may serve as a useful strategy to study the nature of these cells prior to use them for therapeutic efforts.

The aim of the present study was to compare methods for purification, differentiation and culture of putative EPC, which can be further applied to evaluate the role and function of these cells under physiological and pathological conditions. For this purpose we chose a short-term culture of umbilical cord blood mononuclear cells, since effective generation of EPCs has been reported within this time frame employing similar culture methods using MNCs from peripheral blood [5] or bone marrow [13] and cord blood [6]. In addition, an outgrowth assay extending over a 4-week period and using CD34-preselected cells was investigated. This approach, modified from Peichev et al. [10], was chosen because it has been shown to yield a well defined EPC population.

2. Methods

2.1. Isolation of putative EPCs

Mononuclear cells (MNC) were isolated from human umbilical cord blood (HUCB). HUCB samples (about 50 ml each) were collected from fresh placentas with attached umbilical cords by gravity flow. The protocol of the study was approved by the local ethical committee. Heparin was used as anticoagulant. MNC were isolated by density gradient centrifugation over Biocoll (Biochrom, Berlin, Germany) for 20 min at 500 × g and washed three times in PBS (Biochrom). Cells were either selected for the surface marker CD34 using anti CD34-coupled magnetic microbeads (Fa. Miltenyi Biotech, Bergisch-Gladbach, Germany) as indicated by the company or further treated without selection.

2.2. Culture of putative EPCs

Unselected MNC were plated on culture dishes coated with human fibronectin (Sigma, Deisendorf, Germany) and cultured in endothelial cell growth medium (EGM, Clonetics, San Diego, USA) containing EGM SingleQuots: 12 µg/ml bovine brain extract (BBE), 10 ng/ml human
2.3. Phenotypical characterisation of putative EPCs

Cells from MNC cultures were detached with PBS containing 1 mM EDTA and incubated with mouse monoclonal antibodies against VEGFR-2 (Sigma), phycoerythrin (PE)-conjugated CD34 (BD Pharmingen, Heidelberg, Germany) and AC133 (Miltenyi Biotec) and FITC-conjugated CD14, CD45 (Immunotech, Marseille, France), CD105 (Serotec, Düsseldorf, Germany) and VE-cadherin (Bender MedSystems, Vienna, Austria). Antibodies were titrated using cell lines, which did not express the corresponding surface molecule. All incubations were performed at 4°C for 30 min. For the detection of VEGFR-2, cells were further incubated with an isotype-specific FITC-conjugated goat-anti-mouse antibody. Isotype-identical antibodies served as controls to exclude non-specific binding (BD Pharmingen). After incubation cells were washed with PBS containing 0.1% BSA. Quantitative analyses were performed using a FACS SCAN flow cytometer (Becton Dickinson) and CellQuest software (Becton Dickinson).

To further verify that these cells were endothelial progenitor cells, binding of UEA-1, which is specific for human endothelial cells, and uptake of Dil-labelled acetylated LDL, a function associated with endothelial cells, were determined. To detect the uptake of acetylated low-density lipoprotein (Ac-LDL), cells were incubated with 4 μg/ml Ac-LDL labelled with the fluorochrome Dil (Harbor Bioproducts, Norwood, USA) at 37°C for 2 h. Cells were then washed with PBS and fixed with 2% formaldehyde for 10 min. To assess the ability of the putative EPCs to react with endothelial specific lectins, cells were detached by EDTA and incubated with 50 μg/ml FITC-conjugated UEA-1 (Sigma) at 4°C for 1 h. The specificity of UEA-1 binding was tested by inhibition of the lectin binding using 0.2 M α-1-fucose (Sigma) in PBS (data not shown). Incorporation of Dil-labelled Ac-LDL and binding of UEA-1 was determined by FACS analysis.

For analysis of endothelial and progenitor marker proteins of cells from CD34-selected cultures, cells were fixed on days 14 and 28 after isolation with 3.5% paraformaldehyde in phosphate buffered saline (PBS; Biochrom). After permeabilisation with 0.1% Triton X-100 (Sigma) in PBS, cells were stained with fluorescein isothiocyanate (FITC) conjugated monoclonal antibodies (mAb) against CD14, CD34, CD45 (all from Miltenyi Biotech) and CD105 (Serotec, Raleigh, USA), or phycoerythrin (PE) conjugated mAb against CD133 (Miltenyi Biotech). Unconjugated mAb were used to detect von Willebrand Factor (vWF; Dako, Glostrup, Denmark) and VEGFR-2 (T014, kindly provided by Phil Thorpe and Rolf Brekken). Counterstaining was performed with FITC-phalloidin-conjugated goat anti-rabbit serum (Molecular Probes, Leiden, The Netherlands) or with rhodamine isothiocynate (TRITC)-phalloidin (Sigma). As negative controls, different FITC or PE conjugated isotype mAb (Serotec and PharMingen) were used. Subsequently, stained cells were examined using a TNT confocal laser scanning microscope (Leica, Wetzlar, Germany). Three random fields of 20 cells were counted and the average was calculated. Results are given as a percentage of total cell count (20 cells = 100%)
Fig. 1. Putative EPCs from unselected MNC display endothelial and monocytic but not progenitor cell phenotype. Flow cytometric analysis of putative EPCs after 3, 6 and 9 days in culture (A). Cells were labelled with antibodies to VEGFR-2, VE-cadherin, CD105, CD14, CD45, CD34 and CD133. Histograms represent cell number (y-axis) versus fluorescence intensity (x-axis, log scale). Cells (10,000) were acquired and gates were set on living cells. Putative EPCs take up acetylated low density lipoprotein (Ac-LDL) and bind the endothelial specific lectin UEA-1 (B). Incorporation of Dil-labelled Ac-LDL and binding of UEA-1 was determined by FACS analysis. The left panel shows autofluorescence, the right panel specific fluorescence after incubation with Dil-labelled Ac-LDL and FITC-labelled UEA-1. Similar results were obtained in three independent experiments.
CD105 also increased. In addition, cells took up Ac-LDL and bound the endothelial specific lectin UEA-1 (Fig. 1B). Cells were negative for progenitor cell associated markers CD34 and CD133, whereas CD14 and CD45 continued to be expressed, albeit at a decreased level, through day 9.

3.2. Differentiation of CD34-selected putative EPC

In CD34-selected CDNAC culture as described above, the expression pattern of surface markers changed towards a more mature EC phenotype over time (Figs. 2 and 4) as seen by the increasing expression of VEGFR-2 and vWF, with the progenitor phenotype remaining stable as judged by the expression of CD133 and CD34. Simultaneously, CD45 and CD14 expression decreased. CD34-selected CDAC (Fig. 3) showed a strong increase in their expression of VEGFR-2, CD133, CD34 and vWF. Expression of CD14 decreased, while CD45 remained unchanged. The overall level of expression of endothelial- and progenitor cell markers was markedly lower than in CDNAC.

3.3. Cellular morphology of CD34-selected putative EPC

Around 50% of the cells from the original CD34-positive culture remained in the supernatant during the first 3 days of culture (CDNAC) and were transferred to separate culture dishes, where they eventually grew as adherent cells. Both CDNAC and culture-dish-adherent cells (CDAC) did not display a uniform morphology as assessed by light microscopy, indicating the presence of various subpopulations within the CD34-selected cellular fraction (purity 90–95%). Starting from day 10 of culture, more and more cells changed towards a spindle-shaped endothelial-like morphology with a tendency to form lines. Population density increased suggesting active proliferation. Towards the end of the 4-week culture period, more and more cells acquired a round phenotype and lost contact with the culture dish. Thus, the absolute number of vital cells ultimately decreased. These changes were observed in a similar fashion in CDNAC (Fig. 5A,B) as well as in CDAC (Fig. 5C,D), however, fewer cells were present in CDAC cultures and more cells died during the culture period.

4. Discussion

In an attempt to establish a reliable protocol for generation of putative EPC for further research, we have compared previously described protocols for (i) short-term culture of mononuclear cells; and (ii) long-term culture of CD34-selected cells using human umbilical cord blood, a potentially useful source of progenitor cells. We found that all protocols used led to an increase in EC-markers over time. Differences were mainly seen in the overall level of...
expression of these markers as well as in the expression pattern of the progenitor markers CD133 and CD34 and the hematopoietic lineage markers CD45 and CD14.

We found that both unselected MNC and CD34-selected cells from cord blood (CDNAC and CDAC) increased expression of endothelial markers during the time of culture. On day 6 of culture unselected MNC acquired an endothelial lineage phenotype. These cells became positive for VEGFR-2, VE-cadherin and CD105, stained positive for UEA-1 and took up acetylated LDL. Of note, most of the cells expressed the hematopoietic lineage marker CD45 and the monocytic marker CD14. CD14 has also been described to be expressed on putative EPCs derived from adult peripheral blood [13]. MNC failed to express the progenitor cell markers CD133 and CD34. Recently, endothelial progenitors have been described in short-term cultures (7 days) of mononuclear cells derived from HUCB [6]. These cells were positive for the endothelial lineage markers VEGFR-2, VE-cadherin and CD31, stained positive for endothelial constitutive NO synthase, took up acetylated LDL and expressed CD34. Most cells did not express the hematopoietic lineage marker CD45. They formed capillary-like structures on matrigel and augmented neovascularisation when transplanted into the ischemic hindlimb of nude rats. Of note, more putative EPCs could be generated from HUCB than from adult peripheral blood MNCs. Thus, HUCB may be a useful source of putative EPC superior to adult peripheral blood. The endothelial-like cells derived from HUCB-derived MNC described in this paper contrast with those reported by Murohara et al. [6], which were negative for both CD45 and CD14 but positive for CD34. The differences observed may be due to differences in growth factors employed. Murohara et al. used 20% fetal bovine serum supplemented with crude bovine pituitary extract as an EPC growth supplement. We used only 10% fetal calf serum and more defined EPC growth supplements such as VEGF, IGF, EGF and FGF. Since it is poorly understood how specific growth factors affect the selective growth of endothelial-like cells with different expression of surface markers, we cannot pinpoint the observed difference to specific growth factors.

In CD34-preselected cells the progenitor markers CD133 and CD34 were expressed at a fairly high level (about 40%) in CDNAC and remained stable over time whereas their expression in CDAC, which was very low at the beginning, showed a tendency to increase after 4 weeks of culture. Together with the almost complete loss of CD14 in CDAC and the expression of CD14 on MNC during short-term culture, this could indicate transdifferentiation of monocytic cells as previously described [14,15]. It is also conceivable that cells from the monocytic lineage die out over time, thus increasing the prevalence of endothelial-like progenitor cells.

Fifty millilitres of cord blood, which is a volume that can be routinely extracted from one cord, yields about $5 \times 10^6$ EPCs on day 7 of MNC-culture. The number of
CD34+ cells obtained per cord blood was $3.5-7 \times 10^3$. How this relates to the number of cells needed for future therapeutic strategies cannot be predicted, but further efforts are needed to expand these cells in vitro. Investigations on the plasticity of this cell population in vitro or in an in vivo model are of high interest and are the subject of ongoing research.

Our data do not allow a conclusion about coexpression of mature EC-markers, leucocyte markers and progenitor cell markers. However, the lack of a uniform morphology within the cell culture favours the existence of different subpopulations within the CD34-positive cell fraction. Further FACS analysis with double and triple stainings to identify subpopulations within the cell fraction of interest would be required. This, however, is limited by the low number of cells that can be obtained per patient or per cord-blood, respectively.

So far, exact recapitulation of endothelial differentiation from precursor cells in vitro and precise definition of EPC remain elusive. Despite these shortcomings, recent studies...
Fig. 5. CD34-selected putative EPC display a heterogenous morphology during differentiation and eventually die. Cells were seeded in gelatine-coated permanox-chamberslides at a concentration of $1 \times 10^7$ /ml. After 4 days culture-dish-non-adherent cells were removed and further cultured separately. Morphology was examined using phase contrast light microscopy of unstained specimens. Morphological specimens from culture-dish-non-adherent cells (A,B) and culture-dish-adherent cells (C,D) on days 14 and day 28 of culture of one representative experiment are depicted (10-fold original magnification). A reference bar is shown.

have, in principal, already demonstrated the therapeutic usefulness of precursor cells for vascular repair [16], some of them using unselected bone-marrow cells [17].

An important practical consideration is the expandability of endothelial progenitors. Many of the published protocols do not allow expansion. Extrapolating from the EPC numbers injected into mice, the number of EPCs required for therapy in humans exceeds the output of most of the protocols published, at least when systemic delivery is contemplated. In this regard, all protocols using short-term culture will, by their very nature, not yield sufficient numbers of EPC for systemic therapy in the human. Long-term culture, on the other hand, may introduce deviations in the phenotype of the EPC detrimental to their therapeutic efficacy. Furthermore, as already discussed above, the therapeutic use of cells generated from HUCB will most likely be limited by immunological problems.

In conclusion, we report that human EPC as defined by
expression of VEGFR-2 and CD133 can be obtained from HUCB using CD34 selection. These cells can be differentiated in culture to express markers of mature EC, while keeping progenitor markers. Under the conditions described in this paper, cells with endothelial markers can also be generated from unselected MNC. However, their concomitant expression of monocytic and hematopoietic lineage markers and their lack of progenitor cell-associated markers may cast some doubt on their usefulness as true endothelial progenitors. The outgrowth from CD34-selected cells over a 4-week period seems to be superior to short-term culture of unselected mononuclear cells with regard to endothelial lineage-specific differentiation of cells with a premature marker profile.

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References