An affordable method to obtain cultured endothelial cells from peripheral blood

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Abstract

The culture of endothelial progenitor cells (EPC) provides an excellent tool to research on EPC biology and vascular regeneration and vasculogenesis. The use of different protocols to obtain EPC cultures makes it difficult to obtain comparable results in different groups. This work offers a systematic comparison of the main variables of most commonly used protocols for EPC isolation, culture and functional evaluation. Peripheral blood samples from healthy individuals were recovered and mononuclear cells were cultured. Different recovery and culture conditions were tested: blood volume, blood anticoagulant, coating matrix and percentage of foetal bovine serum (FBS) in culture media. The success of culture procedure, first colonies of endothelial cells appearance time, correlation with number of circulating EPC (cEPC) and functional comparison with human umbilical vein endothelial cells (HUVEC) were studied. The use of heparin, a minimum blood volume of 30 ml, fibronectin as a coating matrix and endothelial growing media-2 supplemented with 20% FBS increased the success of obtaining EPC cultures up to 80% of the processed samples while reducing EPC colony appearance mean time to a minimum of 13 days. Blood samples exhibiting higher cEPC numbers resulted in reduced EPC colony appearance mean time. Cells isolated by using this combination were endothelial cell-like EPCs morphologically and phenotypically. Functionally, cultured EPC showed decreased growing and vasculogenic capacity when compared to HUVEC. Thus, above-mentioned conditions allow the isolation and culture of EPC with smaller blood volumes and shorter times than currently used protocols.

Keywords: endothelial progenitor cells ● cell culture ● vasculogenesis

Introduction

Vasculogenesis in adults was for first time reported after the identification and characterization of a population of EPC derived from peripheral blood [1]. Endothelial progenitor cells are incorporated into new vessels undergoing active angiogenesis. This process of postnatal vasculogenesis refers to the formation of new blood vessels from progenitor cells in the adult and has been associated with different physiological functions [2] and pathological disorders as cancer [3], cardiovascular diseases (CVD) [4, 5] and diabetes [6]. Since EPC were discovered, a great deal of literature has emphasized the importance of such progenitor cells in the maintenance of endothelial integrity by both exerting a paracrine effect to promote angiogenesis and integrating themselves into new vessels [6, 7]. Actually, an inverse correlation between EPC numbers and cardiovascular risk factors has been reported in patients with cardiovascular risk, but no history of CVD [8].

Besides the number of EPCs, the progenitor cell function could be affected by individual factors, such as cardiovascular risk factors, different drug treatments, hormone levels and others [9]. The study of EPC function requires obtaining EPC cultures, but one of the drawbacks existing is the lack of a simple and reproducible method to isolate, cultivate and expand EPC. It could be as a result of the small fraction at which these cells are found circulating level, representing only about 0.0001% of total mononuclear cells (MNC) of peripheral blood in human adults [10]. Culture of EPC has been performed in several ways, for early and late EPC [11], although a systematic and
detailed protocol to obtain replicate data on cultured EPC is still required.

Moreover, a potential clinical use of cultured EPC has been proposed, including re-endothelialization of injured vessels, reducing atherosclerotic disease appearance and progression and revascularization of infarcted regions [12]. Endothelial progenitor cells have also been proposed as potential therapeutic tools for gene therapy in growing cancers [13].

In the present study, we evaluated the influence of different culture conditions in obtaining EPC cultures, and we tested EPC functional capacity and compared it with HUVEC, widely used as a laboratory model system for studies on endothelial cell function and pathology. Therefore, we proposed an affordable and reproducible method to obtain EPC cultures to perform functional cell assays.

Materials and methods

Study design

This study was designed to compare different culture conditions for the best way to isolate and to obtain cultured, functional EPC. First, we selected the optimal blood sample withdrawal conditions in terms of volume and anticoagulant. Second, the culture conditions (extracellular matrix, culture media composition) were analysed. Finally, the functional capacity of the cultured EPC (measured in terms of cell growth and adhesion, cell proliferation and vasculogenesis) was the parameter to ensure the best culture conditions of the obtained cells, taking HUVEC as standard reference.

Blood samples

Peripheral blood samples were obtained from 60 healthy individuals at the Cardiology Service, Hospital Clínico of Valencia, in accordance with institutional guidelines. The characteristics of the participants enrolled are presented in Table S1.

Different blood volumes (60 ml) were withdrawn by venipuncture and were collected in tubes containing different anticoagulants: ethylenediaminetetraacetic acid (EDTA), heparin and sodium citrate (Vacutainer, Becton Dickinson, San Agustín del Guadalix, Madrid, Spain). To avoid sample contamination with mature endothelial cells (EC), first 6 ml of collected blood was discarded.

Blood samples were processed within 2 hr after extraction. To assess the effect of time sampling on EPC culture yields, a set of samples were left on a blood roller mixer at room temperature and were processed 24 hrs after withdrawal.

This investigation conforms to the principles outlined in the Declaration of Helsinki, was approved by the Ethical Committee of Clinical Research of the INCLIVA, Hospital Clínico of Valencia, Spain, and written informed consent was obtained from all donors.

Mononuclear cell isolation

Mononuclear cells from peripheral blood samples were isolated as described before [14]. Briefly, non-diluted blood was layered over lymphoprep (Axis-shield, Oslo, Norway) in a volume ratio 2:1, and centrifuged at 800 rcf for 30 min. at room temperature. Mononuclear cells from interphase were collected and washed twice with 6% foetal bovine serum (FBS) (Gibco, Life technologies, Alcobendas, Spain) in Dulbecco’s PBS solution (Gibco).

Endothelial progenitor cell culture

Mononuclear cells isolated from blood samples were collected in endothelial growing media (EGM)-2 complete medium with the following composition (Lonza, Lonza Ibérica, Barcelona, Spain): Endothelial cell basal medium-2 supplemented with EGM Single Quots containing hydrocortisone, 2% FBS, hFGF-B, VEGF, R3-IGF-1, ascorbic acid, heparin, FBS, hEGF, and GA-1000 (gentamicin, amphotericin-B). Mononuclear cells were seeded onto fibronectin-treated plates (2.5 \(\mu g/cm^2\); Becton Dickinson) at a final density of 1.5 \(\times 10^5\) cells/cm². Twenty-four hours after seeding, non-adherent cells were removed and attached cells were further cultured at 37°C and 5% of CO₂. Culture media was changed every 2 days until first EPC colonies appeared or up to a maximum of 40 days.

To test different culture conditions, some of the above-mentioned parameters were modified. In this study, we tested two different FBS media concentrations, 2% and 20%, as well as two different coating matrices, fibronectin 2.5 \(\mu g/cm^2\) and Gelatin 1% (Sigma-Aldrich, Tres Cantos, Madrid, Spain).

Human umbilical vein endothelial cell culture

Human umbilical vein endothelial cell culture was isolated by collagenase treatment of human umbilical veins from newborns as described earlier [15]. Briefly, umbilical veins were flushed with sterile PBS solution (Sigma-Aldrich) to wash the clotted blood out and then perfused with 1% collagenase solution and incubated at 37°C for 15 min. Endothelial cells were recovered by centrifugation and seeded onto gelatin-treated 25 cm² flasks (BioLite, LabClinics, Barcelona) in specific endothelial media EGM-2.

Quantification of circulating EPC in peripheral blood

Peripheral blood samples were recovered in heparin tubes and processed within 2 hrs after extraction. Circulating EPC (cEPC) were stained with anti-human VEGFR2/kinase insert domain receptor (KDR) conjugated with phycoerythrin (PE; R&D Systems, Madrid, Spain), fluorescein isothiocyanate (FITC)-conjugated anti-human CD34 (Becton Dickinson) and peridinin chlorophyll protein complex (PerCP)-conjugated anti-human CD45 (Becton Dickinson) or with the appropriate isotype controls. Red blood cells were lysed (BD FACS Lysing solution, Becton Dickinson) for 10 min. and stained cells were detected with a FC5000 cytometer (Beckman-Coulter, Madrid, Spain) and results were analysed with Infinicyt software (Cytognos S.A., Salamanca, Spain).

Circulating EPC were identified as negative for the leucocyte marker CD45, positive for the prototypical stem cell marker CD34 and positive for the endothelial cell marker KDR (CD45⁻CD34⁺KDR⁺) [11].
Endothelial progenitor cell characterization

Flow cytometry phenotypic characterization
Cultured EPC obtained under the most suitable conditions were also characterized by flow cytometry. The expression of endothelial antigens KDR and CD31 (also known as platelet-endothelial cell adhesion molecule, PECAM-1), progenitor antigen CD34 and leucocyte antigen CD45 was assessed. Briefly, harvested cells were stained with PE-conjugated anti-human KDR, FITC-conjugated anti-human CD31 or FITC-conjugated anti-human CD45, or with the appropriate isotype controls. CD45, CD34 and CD31 antibodies were purchased from Becton Dickinson and KDR antibody from R&D Systems. Stained cells were detected with a FC5000 cytometer (Beckman-Coulter) and results were analysed with Infinicyt software (Cyto- gnos S.A.). Cultured EPC were considered as negative for the leucocyte marker CD45, positive for the stem-cell marker CD34 and positive for the endothelial cell markers KDR and CD31 (CD45−CD34−KDR+CD31+).

Immunofluorescence characterization
The ability of isolated EPC to take acetylated low density lipoprotein (Ac-LDL) and to bind Ulex-lectin, the classical way to define endothelial cells, was performed as described earlier [16,17]. Briefly, EPC were incubated with 1,1′-dioctadecyl-3,3,3′,3′-tetratetramethylindocarbocyanine–labelled Ac-LDL (DiI-acLDL, Life Technologies, Alcobendas, Spain), fixed with 4% paraformaldehyde and incubated with fluorescein isothiocyanate–labelled Ulex europaeus agglutinin (FITC-UEA-1, Sigma-Aldrich). Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). Stained samples were observed on a confocal spectral Leica SP2 microscope (Leica, Barcelona, Spain). Pictures were taken with a 40× objective and shown at ×1200 magnification.

Endothelial progenitor cells cultures were also tested for von Willebrand Factor (vWF) expression. In brief, cells were fixed with 4% paraformaldehyde solution and permeabilized for with PBS 0.05% Triton X-100 solution. Cells were incubated first with rabbit polyclonal vWF antibody (Abcam, Cambridge, United Kingdom) and then with a DyLight 488 conjugated anti-rabbit secondary antibody (Abcam). Counter staining was achieved by incubating cells with DAPI. Images were obtained with an inverted fluorescence microscope Nikon Eclipse Ti (100× magnification).

Functional parameters
Cell growth curve
1.5 × 10^5 cells were seeded in EGM-2 media on each well, culture media was changed daily and the counting process was repeated every day for 6 days. Briefly, cells were detached by using 0.05% trypsin solution, recovered by centrifugation and resuspended with 100 μl of Tripan blue solution (Sigma-Aldrich). Cells were counted on a Neubauer modified chamber and total number of cells per well were plotted to calculate the lag period (latent period of no growth), the log phase (when cells underwent exponential growth), the plateau (when growth rate dropped close to zero) and the saturation density (at which the cell population reached the plateau phase).

Cell proliferation
Cell proliferation was measured by flow cytometry with propidium iodide (PI) staining (Immunostep, Salamanca, Spain) to quantify the content of DNA and the distribution of a cell population along the different phases of the cell cycle [18]. Briefly, cells were starved for 48 hrs and then stimulated for 18 hrs with EGM-2 complete media. After incubation, cells were detached with a 0.05% trypsin solution, fixed with 70% ethanol and stained with PI-RNase solution (Immunostep). Stained cells were analysed with FC5000 cytometer (Beckman-Coulter) and Infinicyt software (Cyto- gnos). Results are shown as percentage of cells undergoing DNA synthesis plus mitosis with respect to total cell number analysed.

Cell adhesion
Adhesion assays were performed on fibronectin (Becton Dickinson) treated dishes (2.5 μg/cm²). In brief, 5 × 10⁴ cells were seeded on dishes with a 4 mm² grid (Nunc, Madrid, Spain) and incubated at 37°C and 5% of CO_2. After 30 min., non-adherent cells were removed and adhered cells were counted in six random squares by two independent observers. Data were expressed as a percentage of adhered cells relative to the total number of seeded cells.

Vasculogenesis assay
Vasculogenesis was analysed in Matrigel (Becton Dickinson) as previously described [19]. In brief, Matrigel was diluted with EGM-2 media SBF free and allowed to solidify for 30 min. at 37°C. Thereafter, 1.5 × 10³ cells/well were seeded and incubated for 8 hrs. Then, pictures were taken with a Nikon Eclipse-Ti inverted microscope (Nikon, Izasa, Valencia, Spain) with 4× objective (total magnification 40×) and images recorded by Nikon digital sight Ds-QiMc camera.

Images from five different random fields per well were processed and analysed with Image Pro-Plus Software V.6 (Media Cybernetics, Rockville, MD, USA). Total length data of the tube-like structures were expressed in micrometers (μm).

Statistical analysis
Values shown in the text and figures are mean ± SEM. For frequency comparison, contingency tables were analysed by Chi-squared test. Data normality was assessed by Kolmogorov–Smirnov test. Statistical comparisons were performed with one-way ANOVA for multiple comparisons and then Bonferroni’s test was performed. Student’s t-Test was applied for single comparisons. Correlation analysis was performed by linear regression. Growth curve data were analysed by two-way ANOVA with Bonferroni's post-test. P < 0.05 was considered significant. The statistical analysis was carried out by using the Prism 5.04 software (GraphPad Software Inc., San Diego, CA, USA).

Results
EPC isolation conditions
Three clinically used anticoagulants were tested to study how the anticoagulant used for blood sampling influences EPC isolation and culture procedure. Identical blood volumes were recovered in tubes containing EDTA, sodium heparin and sodium citrate. Mononuclear cells from these samples were cultured and both the percentage of
EPC cultures and the appearance mean time for the first EPC colonies were recorded.

Only 20% of EDTA-recovered blood samples gave rise to EPC cultures. However, when heparin or sodium citrate was used for blood recovery, 80% of EPC cultures were successfully obtained (P < 0.01; Fig. 1A). No significant differences were found with regard to EPC appearance mean time between anticoagulants (Fig. 1B). Moreover, 100% of cultures derived from citrated samples showed different morphology, were negative for the uptake of Ac-LDL and unable to bind Ulex-lectin, thus demonstrating they were not EPC (Fig. S1). Only heparin allows obtaining high yields of homogeneous EPC cultures with the shorter appearance mean time. Thus, rest of the experiments were performed with blood obtained with heparin as anticoagulant.

To assess the effect of blood volume on EPC isolation procedure, three different blood volumes (10, 20 and 30 ml) were tested. The highest EPC culture yields, referred as the percentage of EPC cultures obtained from blood samples, were found for blood samples of 30 ml (80%; P < 0.05 versus 10 ml). Although there were no statistically significant differences between samples of 20 and 30 ml, the 20% yield reduction in 20 ml blood samples is worth considering (Fig. 1B). The use of higher blood volumes for EPC isolation showed a decrease in EPC appearance mean time, from 16 days for 10 ml blood samples to 12 days for both 20 and 30 ml blood samples (P < 0.05; Fig. 1C). On the basis of obtained results, we chose to collect 30 ml of blood to reach high yields in EPC cultures.

As quick blood processing is not always available, the effect of time sampling on EPC isolation and culture procedure was checked by dividing blood samples into two sets. One set was processed within 2 hrs after extraction. The other set was left in a blood roller mixer for 24 hrs and then processed in the same way (Fig. S2). For samples processed within 2 hrs after withdrawal, 80% of EPC cultures were obtained. For those samples left for 24 hrs in a blood roller mixer, only 30% of EPC cultures were obtained (P < 0.05). These results indicate the critical rapid processing of blood samples when they are intended to EPC isolation.

**EPC culture conditions**

To investigate the role of the most common culture matrixes in the cell culture procedure, MNC were seeded on culture plates previously treated with either gelatin or fibronectin. The highest number of EPC cultures was obtained when fibronectin was used as coating matrix (70% versus 20%; P < 0.05; Fig. 2A). A reduction in the appearance time was also observed, from 23 days for gelatin matrix to 12 days for fibronectin matrix (P < 0.01; Fig. 2B). Therefore, the use of fibronectin matrix for EPC culture increases EPC culture yields as well as it reduces the appearance mean time of the first
EPC colonies. The rest of the experiments were performed onto fibronectin-coated culture surfaces.

Two different concentrations of FBS, 2% and 20%, were tested as complement to endothelial media EGM-2. The higher FBS concentration higher yield in EPC cultures is obtained (30% versus 70%; $P = 0.074$). Endothelial progenitor cells appearance time, however, was significantly reduced from 16 to 11 days ($P < 0.05$) for media supplemented with 2% and 20% FBS, respectively (Fig. 2D).

**Fig. 2** Endothelial progenitor cells’ (EPC) culture conditions. Effect of culture matrix on the development of EPC cultures. Results represent (A) the obtained EPC culture percentages from mononuclear cell (MNC) seeded in plates coated with 1% gelatin or 2.5 $\mu$g/cm$^2$ fibronectin ($P < 0.05$ by Chi-squared test; $n = 10$) and (B) EPC appearance time ($**P < 0.01$ by Student’s $t$-test; $n = 10$). Effect of foetal bovine serum (FBS) media concentration on the development of EPC cultures. Results represent (C) the obtained EPC cultures from MNC maintained with endothelial growing media-2 complete media supplemented with 2% or 20% FBS ($n = 10$) and (D) EPC appearance time ($*P < 0.05$ by Student’s $t$-test; $n = 10$).

Cultured EPC characterization

After isolation and culture procedure, obtained EPC cultures were characterized morphologically. All EPC cultures showed the characteristic endothelial cobblestone morphology. To ensure that our modified procedure gave rise to true EPC, cells were phenotypically characterized by flow cytometry and immunofluorescence microscopy.

Flow cytometry immunophenotyping revealed that all isolated EPC expressed endothelial markers CD31 and KDR. Panleucocyte marker expression, CD45, was absent in all of them and progenitor marker expression, CD34, was found in 45% of cultured cells (Fig. 4A). Taken together, these results suggest our procedure allows the isolation of EPC with endothelial-like phenotype.

The capacity of EPC cultures to incorporate Dil-acLDL and their ability to bind FITC- UEA-1 were analysed in different cultures by confocal microscopy (Fig. 4B). All the cultures were positive for both
markers indicating an endothelial-like phenotype. Moreover, vWF expression was also evaluated by fluorescence microscopy and all the cells were uniformly positive for this marker (Fig. 4C). Therefore, our modified culture procedure successfully achieves the isolation of EPC with endothelial cell-like phenotype.

**EPC functional parameters**

To further confirm the excellence of obtained cultured EPC, the functional ability of EPC in cultures was evaluated in terms of cell growth kinetics, proliferation, adhesion and vasculogenesis. EPC functional parameters were compared with those obtained for HUVEC, the well-accepted endothelial cell model for studies in vitro.

Endothelial progenitor cells and HUVEC growth curves are presented in Figure 5A. Cells were counted every day until cells reached confluence. Endothelial progenitor cells showed longer lag phase (2.4 days) compared with HUVEC (1.3 days; $P < 0.05$). Endothelial progenitor cells cultures were found to have shorter exponential growth phase. Endothelial progenitor cells plateau growth phase was reached on the 4th day after seeding at a saturation density of $5.2 \times 10^5$ cells/cm$^2$. By contrast, HUVEC reached plateau growth phase on the 5th day after seeding at a saturation density of $17.7 \times 10^5$ cells/cm$^2$ ($P < 0.001$; Fig. 5A).

Cell proliferation was studied both in starvation and after stimulation with complete EGM-2 media, after 48 hrs of starvation (Fig. 5B). In starved conditions, about 10% of cells were proliferating. When cells were stimulated with complete media, the percentage of cells actively involved in proliferation was increased four- to sixfold ($P < 0.05$ versus starvation). No differences in proliferation capacity between EPC and HUVEC cultures were found under starvation conditions or under induced conditions.
The cell adhesion function was evaluated by the cell ability to adhere to an extracellular matrix of fibronectin for 30 min. Results showed no differences between EPC and HUVEC (32% versus 35%, respectively; Fig. 5C).

Vasculogenesis, the ability to form tube-like structures, was assessed in vitro by seeding the cells on Matrigel matrix. All EPC and HUVEC cultures were able to organize themselves into similar tube-like structures (Fig. 5D). After 8 hrs of incubation, total length for these structures was measured. Human umbilical vein endothelial cells cultures formed larger tube-like structures (664 ± 25 μm) when compared with those formed by EPC cultures (394 ± 92 μm; P < 0.05; Fig. 5E).

**Discussion**

This study standardizes the most controllable conditions for optimal isolation and culture of EPC from peripheral blood samples, the less invasive source for EPC available so far. Our results point to the need to control a number of parameters related to the sample collection and to the culture conditions to obtain an optimal functional performance of EPC. The conditions tested indicate (i) peripheral blood should be recovered in heparin tubes; (ii) in a minimum volume of 30 ml; (iii) blood samples should be processed within 2 hrs after collection; (iv) fibronectin is the best extracellular matrix and (v) culture media should be supplemented with 20% FBS.

Knowledge of EPC biology represents an advance in the understanding of vascular repair mechanisms in physiological and pathological situations. Furthermore, the fact of being able to successfully isolate and culture EPC makes us closer to test them for potential clinical applications in regenerative and anticancer therapies [12, 13].

In this regard, the use of standard procedures for isolating and culturing EPC is the only way to obtain comparable results. Different culture procedures could lead to the isolation of different cell types.
with very different properties. A good example of this fact is the great
difference in morphology, phenotype and behaviour between the so-
called early and late EPC where time is the main difference condition
for EPC culture and isolation (for a review, see [20]). Because of the
complex procedures for EPC isolation and culture, the comparison of
different works is quite challenging if not sometimes impossible.
There is a lack, however, of a methodical comparison of different
parameters for EPC isolation and culture.

Blood recovery and handling are an extremely important step in
EPC isolation as anticoagulants and sample timing handling may
affect EPC integrity. In some cases, other anticoagulants have been
used, although their effect on EPC isolation and culture yields, to our
knowledge, has not been systematically compared. Our results sug-
gest that correct sample handling would involve the use of heparin as
anticoagulant.

An important variable when using human samples, especially to
apply the procedure to patients in different circumstances, is the time
spent between blood sample collection and its processing. In our
study, a sample process time inferior to 2 hrs after withdrawal is
mandatory to obtain the highest EPC culture yields.

Regarding culture conditions, two important parameters demon-
strated an important impact on the probability to obtain an EPC cul-
ture and the mean appearance time. On the one hand, the use of
fibronectin as matrix to coat cell culture surfaces triplicated the
success and half-reduced the appearance time, in agreement with
previous reports where EPC showed a greater adherence on fibronect-
in matrix than collagen [1]. On the other hand, the FBS, which is
commonly added to cell culture media as a source of growth factors,
cytokines and essential nutrients, is also critical to increase the likeli-
hood to obtain EPC cultures and to reduce the EPC appearance mean
time.

As previously reported, the volume used to isolate EPC from
peripheral blood samples ranged from 50 to 100 ml [14, 21]. How-
ever, a minor volume of blood sample of 20 ml was required when
the origin was human umbilical cord blood samples [14] probably
because of a higher content in progenitor cells. Taken together, our
results suppose a reduction in blood sample volumes and culture
mean time than those found earlier for peripheral blood samples and
a significant increase in the success rates (compared to 13% achieved in other studies [22]).

Circulating EPC levels change with several physiological condi-
tions, such as menopause [23], physical training or ageing [24].
Acute disorders, such as myocardial infarction [25] or vascular
trauma [26], increase the number of circulating EPC in blood sam-
ples. Conversely, an inverse correlation between EPC number and risk
factors has been described in coronary artery disease [27].

The present study demonstrated an inverse correlation between
the number of cEPC and EPC appearance mean time in culture,
suggesting that the higher the number of circulating EPC in blood
samples, the higher is the likelihood and the shorter the time to
successfully obtain EPC cultures out of them. This information is
of great importance if our proposed procedure is intended for
EPC isolation, culture and cell therapy. In this case, previous EPC
mobilization would be strongly recommended to ensure high
yields and time reduction in EPC cultures, particularly in those
pathologies where EPC numbers have been reported to be reduced
[27].

But not only is the yield of the method important but the func-
tional characteristics of the obtained EPC. The morphological, pheno-
typical and functional characteristics of cultured EPC were
comparable to endothelial cells from other sources, such as HUVEC.
Cultured EPC showed a cobblestone-like morphology was positive for
LDL uptake and Ulex-lectin binding, expressed progenitor antigen
CD34 as well as endothelial antigens CD31, KDR, vWF while lack the
expression of hematopoietic marker CD45. Our procedure give rise to
EPC with similar traits to the so-called late EPC, endothelial colony
forming cells or endothelial outgrowth cells [11, 14, 20, 21].

Endothelial progenitor cells obtained under the best conditions
exhibited functional parameters comparable to those obtained for
HUVEC in terms of growth curve, cell proliferation and adhesion
and vasculogenesis. In spite of cultured EPC showed reduced
growth (which can be attributed to the longer lag phase) and vas-
culogenic capabilities than HUVEC, no differences were found in
proliferative and adhesive capabilities between these two cell types.
Therefore, the culture conditions provided are not only an adequate
tool to study EPC but also a source of endothelial cells from
adults.

The agreement on EPC definition (CD34+KDR+ phenotype) repre-
sents the best compromise in terms of detection accuracy, biological
meaning and clinical usefulness [28], as well as the development of
standardized procedures for EPC isolation and culture opens the use
of EPC for clinical application.

In summary, we presented a standard protocol for EPC isolation
and culture from human peripheral blood. Additional in vivo testing of
the isolated EPC following our procedure must be performed to fully
characterize the possible potential of this cell population for cell ther-
apy use and regenerative medicine.

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Conflicts of interest

The authors confirm that there are no conflicts of interest.

Supporting information

Additional Supporting Information may be found in the online
version of this article:

Figure S1 Phenotypic characterization of cells isolated from citrate
tube collected blood. Bright field images of cell cultures (A) ×40 and
(B) ×200 magnification. Fluorescence microscopy of DiI-Ac-LDL uptake (C), FITC-UEA-1 binding (D), DAPI nuclei staining (E) and merged images (F) are shown. Cells were incubated with 2 μg/ml of Ac-LDL for 1 hr, fixed with 4% paraformaldehyde and then incubated with 10 μg/ml FITC-Ulex-lectin. Counterstaining was achieved by 1 μg/ml DAPI staining. Scale bar represents 100 μm (original magnification for fluorescence microscopy images: ×200).

Figure S2 Influence of processing time on the success of EPC cultures. Blood samples were divided into two sets. One was processed within 2 hrs and the other 24 hrs after withdrawal. The success of EPC culture was expressed as the percentage of EPC cultures obtained (*P < 0.05 by Chi-squared test; n = 10).

Figure S3 MNC culture and EPC isolation. Representative images of EPC cultures. (A) MNC after seeding. After 24 hrs of incubation, non-adhered cells were removed and attached cells (B) were further cultivated. On day 15 of culture, first EPC colonies appeared (C). EPC colonies were cultured for 7 days or until they reached confluence (D). All the pictures above shown were taken at ×100 magnification.

Table S1 Characteristics of healthy individuals.