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Comparison Between Total Endothelial Progenitor Cell Isolation Versus Enriched Cd133$^+$ Culture

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Endothelial progenitor cells (EPCs) play a role in endogenous neovascularization of ischaemic tissues. Isolation and characterization of EPCs from circulating mononuclear cells are important for developing targeted cellular therapies and reproducibility of data are the major scientific goals. Here we compared two currently employed isolation methods, i.e. from total peripheral blood mononuclear cells (PBMCs) and from enriched CD133$^+$ cells, by defining the cell morphology and functional activities. We show that EPCs from cultured PBMCs resulted in an adherent population of 23% ± 4% merged cells positive for Dil-Ac-LDL and lectin, whereas the percentage of double positive cells in cultured CD133$^+$ enriched cells was 50% ± 7% ($P < 0.01$). These data were obtained through a novel and a more complete method of analysis of cell calculations (specifically by dividing each microscope field into 120 subfields). When stimulated with tumour necrosis factor α (TNF)-α and glucose, cell number was reduced in EPCs from total PBMCs and, more consistently, in CD133$^+$ enriched cells. However, both cultured total PBMCs and CD133$^+$ enriched cells respond similarly to TNF-α or glucose-induced p38-phosphorylation.

EPCs from both procedures show similar results in terms of phenotype and response to modulators of their functional activities. However, when the cell phenotype of CD133$^+$ enrichment-derived cells was compared with that of cells from the total PBMC, a significant increase in CD133$^+$ expression was observed ($P < 0.01$) This may have relevance during intervention studies using cultured EPCs.

Key words: CD133, endothelial progenitor cells, glucose, TNF.

Abbreviations: Dil-Ac-LDL, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanina-labeled acetylated LDL; EC, endothelial cells; EPCs, Endothelial progenitor cells; KDR, kinase insert domain receptor; MAP, kinase, mitogen activating protein kinase; PBMCs, peripheral blood mononuclear cells; PE, phycoerythrin; TNF-α, tumor necrosis factor-alpha; VE-cad, VE-cadherin; VEGF, vascular endothelial growth factor; VEGFR-2,VEGF-receptor-2.

Isolation, differentiation and expansion of endothelial progenitor cells (EPCs) from peripheral blood have potential applicability in areas of therapeutic neovascularization, vascular repair and tissue engineering (*1–5*). The postulated rationale of this action is due to EPC homing at the sites of vascular damage (*6*, *7*). Some published studies used EPCs from total peripheral blood mononuclear cells (PBMCs) as standard method to explore their pathophysiological characteristics in the context of immunological markers typical of these cells (*7*). However, some methodological concerns are still raised in order to select the gold and immunogenic standard properties of EPCs, for example, adult cultured EPCs maintained monocytoid function throughout cell culture (*8*).

Although EPCs are usually derived from PBMCs cultured in presence of vascular endothelial growth factor (VEGF) and identified as a population of adherent cells with both 1,1′-dioctadecyl-3,3,3′, 3′-tetramethylindocarbocyanine-labelled acetylated LDL (Dil-Ac-LDL) uptake and lectin binding, several methods of isolation are currently described (*8*, *9–15*). To generate putative EPCs, short-term culture of PBMCs over 4–7 days leads to adherence and differentiation of putative EPCs defined by the expression of VEGF-receptor-2 (VEGFR-2), vWF, VE-cadherin and CD31 (*13*). Other methods involve selection through surface markers such as CD133 (*9*, *10*, *14*), VEGFR-2 (*16*), and CD34, another so-called stem cell marker expressed at a very early developmental stage (*11*, *12*). Although CD34$^+$ or CD133$^+$ progenitors purified by the immunomagnetic technique represent a very small subset of PBMCs, they

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can generate endothelial cells and exhibit revascularization properties *in vivo* (*17*). A major source of circulating EPCs has been described as a subset of a double positive $CD14^+CD34^{low}$ (*18*). Conventional cytofluorimetric techniques of PBMC-derived EPCs have shown that these cells consist of a population mainly derived from the monocyte/macrophages-containing $CD34^-$ mononuclear cell population (*19*) and only in part from the haematopoietic stem cell-containing $CD34^+$ mononuclear cell population (*20*, *21*). At the same time, adherence-related selection of cultured PBMCs allows for the recovery of a number of EPCs sufficient for therapeutic treatments (*3*, *6*, *7*, *17*, *22*) suggesting that EPCs can also originate from circulating populations other than $CD34^+$ or $CD133^+$ progenitors. Thus, since the exact origin of EPCs is still not clear the culture of pre-selected PBMCs could preclude certain type of EPCs. The procedures currently used allow the isolation of two main types of EPCs: EPCs that coexpress monocyte/macrophage and EC markers or EPCs that express only typical EC markers and show high proliferating activity (*23*, *24*).

To date, published studies were not homogeneous in terms of methodology employed and reproducibility. Thus, the aim of the present study was to compare the two principal methods for isolation and culture of EPCs and to elucidate whether differences in the evaluation of their role and function under pathophysiological conditions can vary upon the isolation method used. For this purpose we compared the short-term culture of unprocessed PBMCs and $CD133^+$ enriched cells.

METHODS

Regular EPC Isolation and Cultivation—EPCs were isolated from total PBMCs as previously described (*25*). Briefly, PBMCs were isolated by density gradient centrifugation ($400 \times g$ for 40 min at 4°C) of 15 ml of leucocyte-rich buffy coat of healthy human donor on 20 ml of Histopaque-10771 (1.077 g/ml, Sigma). After centrifugation the interface cells were carefully removed and transferred to a new conical tube. Cells were washed twice with Pipes (1×), centrifuged at $300 \times g$ for 10 min at 4°C and then suspended in 9 ml of H_2O, 3 ml KCl 0.6 M to a final volume of 50 ml of Pipes (1×). After centrifugation at $300 \times g$ for 10 min at 4°C the pellet was suspended in an appropriate volume of Pipes (1×) and cells were counted. Isolated PBMCs ($\sim 200 \times 10^6$ cells) were plated on culture dishes (5×10^6 cells/ml medium) coated with human fibronectin and maintained in endothelial basal medium (EBM; Cell Systems) supplemented with 1 μg/ml hydrocortisone, 12 μg/ml bovine brain extract, 50 μg/ml gentamycin, 50 ng/ml amphotericin B, 10 ng/ml epidermal growth factor, and 20% FCS (*25*). Cells were cultured at 37°C with 5% CO_2 in a humidified atmosphere for 3 days. After 3 days of culture a low percentage of cells was attached (~10% of the total plated PBMCs). The nonadherent cells were removed by washing with PBS and adherent cells were used for further analysis.

Enriched Procedure of $CD133^+$ EPC Isolation and Cultivation—PBMCs were used to isolate EPCs with immunomagnetic CD133-bound microbeads (Miltenyi Biotec) following the manufacturer's protocol (100×10^6 cells PBMCs/column). Briefly, the magnetically labelled the $CD133^+$ cells were retained in a LS column and subsequently eluted as the positively selected cell fraction after removal of the column from the magnetic field. Isolated $CD133^+$ enriched cells (2×10^6 cells) were plated on culture dishes coated with human fibronectin (0.25×10^6 cells/ml medium) and maintained in complete EBM (*25*). Cells were cultured at 37°C with 5% CO_2 in a humidified atmosphere. After 3 days of culture, at the first media change, all seeded cells were adherent.

Dil-Ac-LDL/Lectin Staining and Cell Calculation Method—Total PBMCs (5×10^6 cells/ml medium) or enriched $CD133^+$ cells (0.25×10^6 cells/ml medium) cells were grown on microscope fibronectin coated glasses in 24-multiwell plates for 3 days.

After 3 days of culture, non-adherent cells from total PBMC preparation were removed by washing with PBS. EPCs isolated from total PBMCs and $CD133^+$ enriched cells were incubated with 2 μg/ml 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-labelled acetylated LDL (Dil-Ac-LDL) (Biomedical Technologies Inc.) for 3 h at 37°C as previously described (*25*, *8*). Cells were fixed in 4% paraformaldehyde and counterstained with 50 μg/ml FITC-labelled lectin from *Ulex europaeus* (Sigma) for 1 h at 37°C in the dark. Then, 3–5 power fields were randomly counted using a computer-based program (Leica FW4000). Nuclear staining was performed by Hoechst 33258 (4 μg/ml) (Sigma). Cell counting was performed by using Photoshop software, in which the cells from the each field to be counted can be marked (*8*), and, more accurately, by dividing each microscope field image in 120 subfields by an array. Total number of double positive Dil-Ac-LDL/Lectin cells was calculated by counting cells in each subfield. EPC number was expressed as percentage of cells positive for Dil-Ac-LDL/Lectin dual staining. EPC distribution in the microscope field was monitored by dividing total double positive Dil-Ac-LDL/Lectin cells for the number of sub-fields, excluding empty subfields.

Flow Cytometry Analysis—Flow cytometry analysis (FACS) was performed on freshly isolated cells at (day 0) and on the cells after 3 days of culture (day 3). Briefly, the freshly isolated cells, 10^6 total PBMCs and 10^5 enriched $CD133^+$ cells, were washed with PBS, resuspended with PBS/BSA 0.1% and then incubated at 4°C in the dark for 1 h with directly conjugated mouse monoclonal antibodies to CD34-phycoerythrin (PE) or VE-cadherin-PE (Santa Cruz) or for 10 min with directly conjugated mouse monoclonal antibodies to CD133-PE (Miltenyi Biotec). A PE isotype-matched antibody was used as negative control. The cells were then washed twice with PBS/BSA and fixed with PBS/FBS-2%/PFA-2% for 10 min at room temperature and analysed in PBS/BSA. Quantitative fluorescence analysis was performed with a FACS-CANTO instrument (BD Biosciences). Each analysis included 10,000 events.

Cell Treatments—EPCs from total PBMCs or $CD133^+$ enriched cells were incubated with tumour necrosis factor α (TNF)-α (10 ng/ml) or glucose (15 mmol/l) for

3 days (day 0–3) without changing the medium or at day 3 (day 3) for 10 min as described (*25*). After 3 days of culture, cell morphology and TNF-α or glucose-induced-reduction of EPC number and activation of p38 MAP kinase were determined (*25*).

Western Blot Analysis—Total cell extracts (20–50 μg/lane) were loaded onto SDS-polyacrylamide gels and blotted onto polyvinylidene difluoride membranes. Western blots were performed by use of antibodies directed against phospho-p38 (Thr180/Tyr182)(3D7) (1:1000; cell signalling), total p38 (c-20) (1:1500; Santa Cruz Biotechnology), tubulin (GTU-88) (1:10,000; Sigma). Secondary antibodies were anti-rabbit (1:4000; Santa Cruz Biotechnology) and anti-mouse antibody (1:5000; Santa Cruz Biotechnology). Enhanced chemiluminescence was performed according to the instructions of the manufacturer (Amersham). The autoradiographs were scanned and semi-quantitatively analysed. The protein ratio was calculated by LKB analyzer.

Statistical Analysis—Data are given as mean ± SD. Differences were assessed by *T*-test and a *P*-value < 0.05 was considered to be significant.

RESULTS

EPC Morphology—Culturing human total PBMCs (5×10^6 cells/ml medium) for 3 days under standard conditions (*25*) resulted in an adherent population consisting of ~10% of the total plated PBMCs ($\sim 0.5 \times 10^6$ cells/ml medium). Phase control fluorescent microscope counting indicated that 23% ± 4% of these cells were double positive for Dil-Ac-LDL (red) and lectin (green) (Fig. 1a), thus matching the previously described EPC phenotype (*21*). When EPCs were isolated from total PBMCs (100×10^6 cells PBMCs/column) by immunomagnetic $CD133^+$ selection the yield was ~1–2%. However, fluorescence microscopy cell counting indicated that the percentage of Dil-Ac-LDL/Lectin positive cells in cultured $CD133^+$ enriched cells (Fig. 1b) was consistently higher than that obtained with total PBMC procedure (50% ± 7% of positive cells compared with 23% ± 4% double positive cells from total PBMCs). Cell counting was performed by dividing microscope field image in 120 subfields (Fig. 1c and d) and total number of double positive Dil-Ac-LDL/Lectin cells was calculated by counting cells in each subfield (Fig. 1c). EPC number was expressed

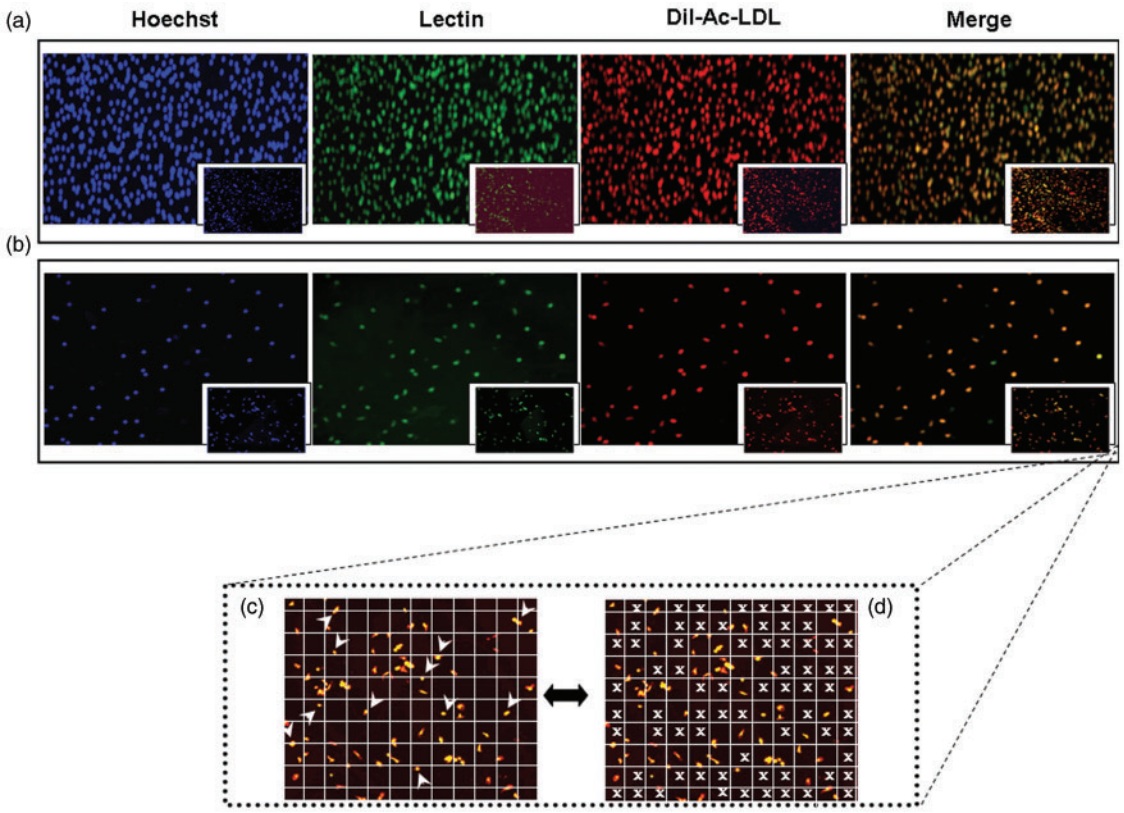

Fig. 1. **Characterization of EPCs.** Dil-Ac-LDL uptake and lectin binding of isolated EPCs were determined by fluorescence microscopy (a) from PBMCs and (b) from $CD133^+$ enriched cells. Hoechst positive nuclei from PBMCs and $CD133^+$ enriched cells were 453 ± 86 cells/field and 94 ± 21 cells/field, respectively. Overlay images are shown in right panel. Cell counting was performed by dividing microscope field images in 120 subfields by an array (c, d). (c) Total number of double positive Dil-Ac-LDL/Lectin cells was calculated by counting cells in each subfield and was expressed as percentage of EPC positive for merged Dil-Ac-LDL/Lectin dual staining. (d) EPC distribution was monitored by dividing total double positive Dil-Ac-LDL/Lectin cells for the number of subfields, excluding empty subfields. Two different experiments are shown, one as main image and another as insert. Data are expressed as mean ± SD. Images are representative of three different experiments in duplicate.

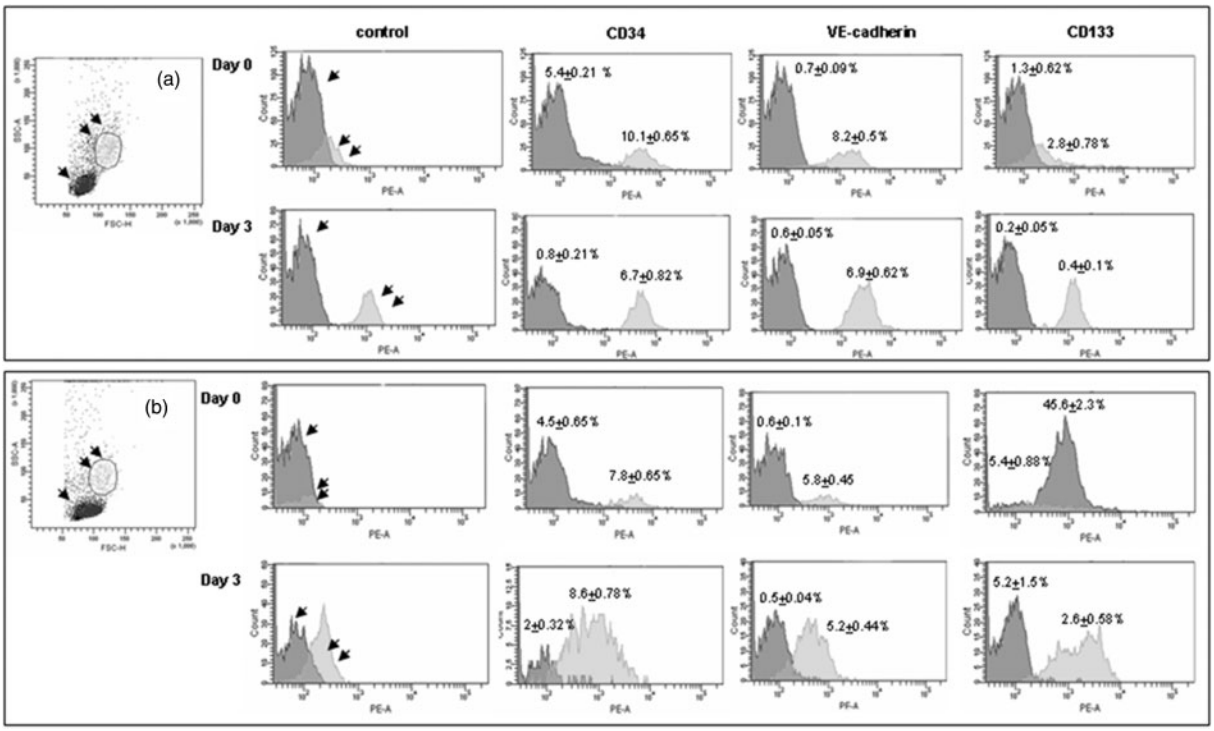

Fig. 2. **Flow cytometry analysis of PBMCs and CD133$^+$ enriched cells.** (a) PBMCs were obtained by density gradient centrifugation of leukocyte-rich buffy coat on Histopaque-1077 as described. Expression of surface markers was performed on PBMCs freshly isolated (day 0) and after 3 days of culture (day 3); (b) CD133$^+$ enriched cells were separated from PBMCs as described and analysed for expression of surface markers on cells freshly isolated (day 0) and after 3 days of culture (day 3). Controls were corresponding to negative isotype controls. Results are representative of 3 separate experiments in duplicate. Data are expressed as mean ± SD.

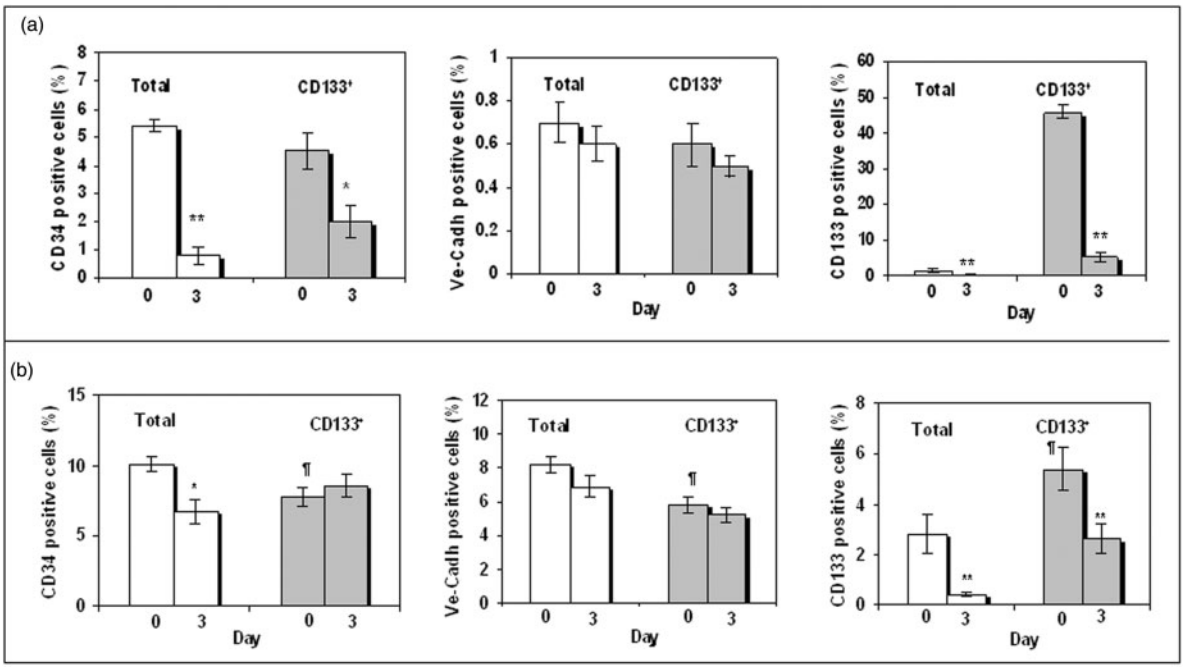

Fig. 3. **Phenotype of PBMCs and CD133$^+$ enriched cells during culture.** Phenotype of total PBMCs and CD133$^+$ enriched cells was analyzsed during short-term culture in (a) lymphocyte gated population and (b) monocyte gated population. Data are expressed as mean ± SD ($n = 3$). $^*P < 0.05$ versus day 0. $^{**}P < 0.01$ versus day 0. $^¶P < 0.05$ of CD133$^+$ enriched cells at day 0 versus total PBMCs at day 0.

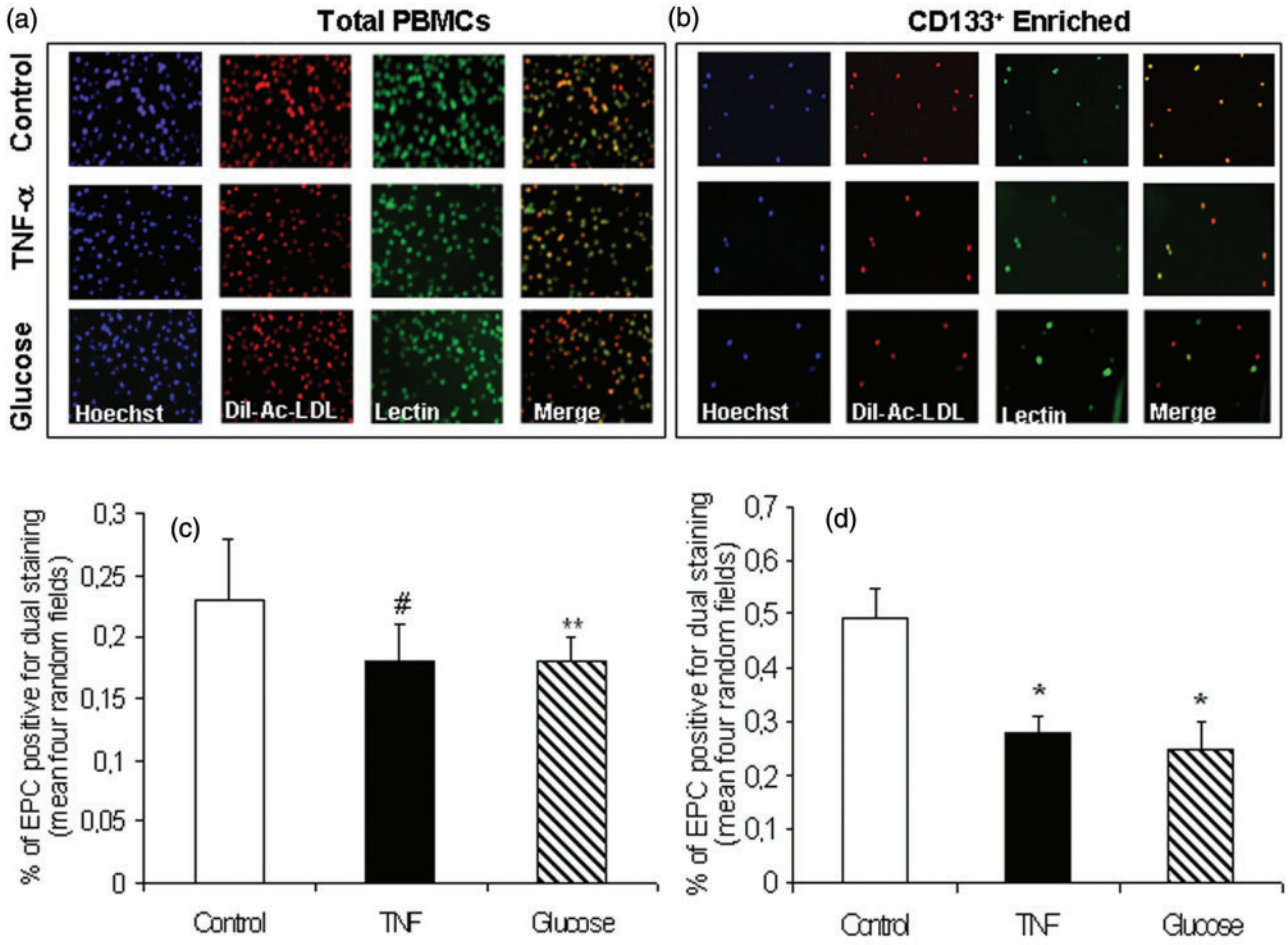

Fig. 4. **Effect of TNF-α and glucose on EPC number isolated by two compared methods.** Dil-Ac-LDL uptake and lectin binding staining of isolated EPCs were determined by fluorescence microscopy (a) from PBMCs (regular) untreated and treated with TNF-α and glucose; (b) from $CD133^+$ enriched cells (enriched) untreated and treated with TNF-α and glucose; (c) bar graphs of corresponding EPC numbers from PBMCs (regular) and (d) $CD133^+$ enriched cells (enriched) (untreated and treated with TNF-α and glucose), respectively. Treatments with TNF-α and glucose were performed as described. Hoechst positive nuclei in control, TNF-α, and glucose samples prepared from total PBMCs were 148 ± 15 cells/field, 104 ± 18 cells/field and 140 ± 25 cells/field, respectively. In samples prepared from $CD133^+$ enrichment nuclear Hoechst positive cells in control, TNF-α, and glucose were 12 ± 3 cells/field, 7 ± 1.5 cells/field and 8 ± 2 cells/field, respectively. Results are expressed as mean ± SD and are representative of three different experiments in duplicate. Differences were assessed by T-test. $^*P < 0.01$ versus untreated; $^{**}P < 0.05$ versus untreated; $\#P > 0.05$ versus untreated.

as percentage of cells positive for Dil-Ac-LDL/Lectin dual staining. Moreover, EPC distribution in the microscope field was monitored by dividing total EPC number for the number of subfields, excluding empty subfields (Fig. 1d). After 3 days, most of the cells appeared elongated and became spindle-shaped in $CD133^+$ enriched preparation whereas round cells were still present in the cell population from total PBMCs.

Surface Marker Expression of PBMC and $CD133^+$ Enriched Cells—When we isolated PBMCs from leukocyte-rich buffy coat, we found that cells were positive for stem/progenitor cell surface markers CD34 and CD133, which means that freshly isolated PBMCs contain the cells from which the EPCs originated. This was true also for the expression of endothelial markers, VE-cadherin (VE-cad) (Fig. 2a–b). As indicated by the forward and sideward scatter, two main populations were gated, corresponding to the lymphocyte gate (single arrow) and to the monocyte gate (double arrow). In freshly isolated cells (day 0) the percentage of lymphocyte gated cells expressing CD34 and VE-cad did not significantly differ between total PBMCs and $CD133^+$ enriched cells (Fig. 3a). However, as expected, a significant difference in the percentage of cells expressing CD133 was observed ($1.3\% \pm 0.6\%$ in total PBMCs compared with $45.6\% \pm 2.3\%$ in $CD133^+$ enriched cells) ($P < 0.01$). The monocyte gated population of freshly isolated total PBMCs (day 0) showed a higher percentage of cells expressing CD34 and VE-cad compared with $CD133^+$ enriched cells (Fig. 3b). Similarly to the lymphocyte gated population, the monocyte gated population of $CD133^+$ enriched cells showed a higher percentage of CD133 positivity compared with total PBMCs.

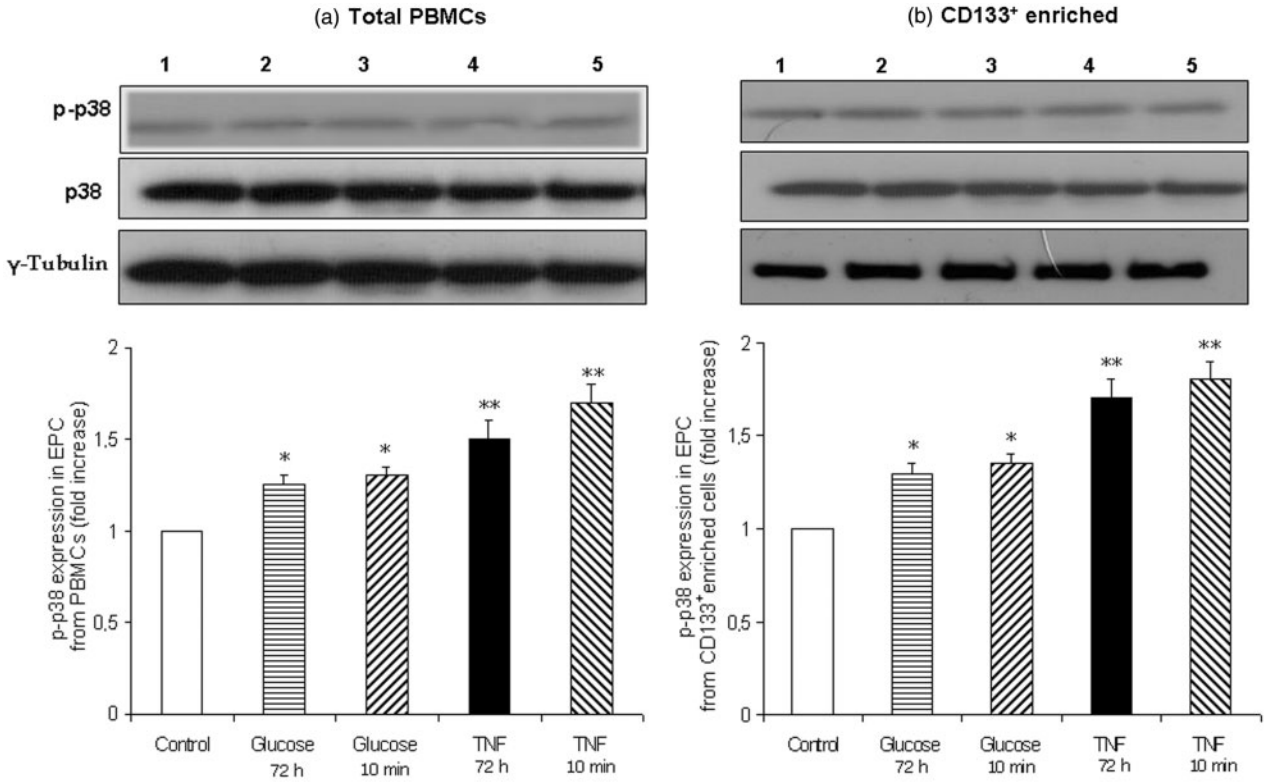

Fig. 5. **Detection of p38 phosphorylation.** (a) PBMCs and (b) $CD133^+$ enriched cells were treated with or without TNF-α (10 ng/ml) or glucose (15 mmol/l glucose) from day 0 to day 3 or at day 4 as a stimulation for 10 min as described. γ-tubulin and total p38 served as loading controls. Lanes 1 are control cells; lanes 2: cells treated with glucose for 72 h; lanes 3: cells treated with glucose for 10 min; lanes 4 are cells treated with TNF-α for 72 h; lanes 5 are cells treated with TNF-α for 10 min. Data are mean ± SD and are representative of three different experiments, $^*P<0.05$ versus untreated, $^{**}P<0.01$ versus untreated. Immunoblotting images are representative of three Western blotting experiments. Densitometry analysis was performed for p-p38 lanes.

When the phenotype of total PBMCs and $CD133^+$ enriched cells was analysed after 3 days of culture significant changes were observed in the two gated populations. The lymphocyte gated population of both total PBMCs and $CD133^+$ enriched cells showed a significant decrease of the CD34 and CD133 positive cells ($P<0.05$ or $P<0.01$). In contrast, the cell phenotype of the monocyte gated population of both total PBMCs and $CD133^+$ enriched cells showed a significant decrease only of the CD133 positive cells percentage ($P<0.05$ or $P<0.01$). A significant decrease of the percentage of CD34 positive cells was observed in total PBMCs ($P<0.05$) but not $CD133^+$ enriched cells. Finally, both EPCs from total PBMCs and $CD133^+$ enriched procedure exhibited a constant expression of VE-cad during short-term culture.

Modulation of EPC Number Derived from Total PBMCs and $CD133^+$ Enriched Cells—Stimulation with TNF-α and glucose impairs EPCs functional activity reducing cell number (*25*). Both forms of EPCs were incubated with TNF-α (10 ng/ml) or glucose (15 mmol/l) for 3 days (day 0–3) accordingly to previous experiments (*25*). Adherent cells, that were double positive for both lectin and Di-LDL uptake, were counted at day 4. As shown in Fig. 4 (panels A–D), the inhibitory effect of TNF-α and glucose on EPC number was observed both in EPCs from PBMCs and from $CD133^+$ enriched cells, although it was more evident on $CD133^+$ enriched cells (with a *P*-value in the latter case <0.01 versus a *P*-value <0.05 for glucose treatment and $P>0.05$ for the TNF-α treatment).

p38 MAP Kinase Activation in EPCs Derived from Total PBMCs and $CD133^+$ Enriched Cells—The mechanism underlying the effect of TNF-α or glucose on EPC number involves p38 MAP kinase (*25*). An increased p38-phosphorylation was observed in TNF-α - or glucose-treated EPCs (Fig. 5). Densitometry analysis revealed that total p38 was uniformly expressed in EPCs derived from total PBMCs (Fig. 5a) and $CD133^+$ enriched cells (Fig. 5b) and that no significant differences in p38 phosphorylation were observed between treated-EPCs obtained by both methods ($P<0.01$ after TNF-α treatment and $P<0.05$ after glucose treatment versus either untreated cells from total PBMCs and untreated $CD133^+$ enriched cells).

DISCUSSION

We show that EPCs isolated from previously described isolation protocols, unprocessed total PBMCs and $CD133^+$ enrichment, show similar phenotype and morphologic characteristics, and respond similarly to modulators of their functional activities. EPCs from both

Table 1. **Phycoerythrin -mean fluorescence of PBMCs and CD133$^+$ enriched cells markers expression during their culturing to obtain EPCs.**

	Control	CD34	VE-Cadh	CD133
PBMCs				
Lymphocyte gate (PE-mean fluorescence)				
Day 0	81 ± 8.2	1455 ± 12.2	704 ± 7.4	968 ± 6.5
Day 3	76 ± 6.5	892 ± 6.3	470 ± 5.5	567 ± 5.3
Monocyte gate (PE-mean fluorescence)				
Day 0	179 ± 3.1	5092 ± 41.5	4858 ± 10.2	3293 ± 21.2
Day 3	698 ± 4.2	1898 ± 11.5	1784 ± 9.2	671 ± 5.3
CD133$^+$				
Lymphocyte gate (PE-mean fluorescence)				
Day 0	75 ± 5.2	1467 ± 10.3	445 ± 10.5	9471 ± 88.2
Day 3	82 ± 6.2	980 ± 10.2	341 ± 12.1	1257 ± 15.8
Monocyte gate (PE-mean fluorescence)				
Day 0	92 ± 10.5	1912 ± 54.3	1569 ± 61.2	1531 ± 47.6
Day 3	214 ± 22.3	5312 ± 75.2	1228 ± 33.5	992 ± 61.8

total PBMCs and CD133$^+$ enrichment showed the attached spindle-shaped cells that have been regarded as EPCs (*22*). These cells did not develop in cluster and reach confluence during the short-term culture.

EPCs obtained by unprocessed total PBMCs culture show morphological signs of endothelial markers starting between day 3 and 7 of culture (*22*, *25*). At the same time, CD133$^+$ progenitor cells from peripheral blood differentiate into adherent endothelial progenitors with both haematopoietic and endothelial character (*9*). In the attempt to define whether cellular response might differ among EPCs isolated with different methods, we have compared previously described protocols for short-term culture of total PBMCs and CD133$^+$ enriched cells. When the phenotype of cells derived from CD133$^+$ enrichment was compared with that of cells from the total PBMC, we found a significant increase in CD133$^+$ expression (45.6% of CD133 positive cells compared with 1.3% in total PBMCs, $P < 0.01$) and a 2-fold increase of the percentage of double positive cells for Dil-Ac-LDL uptake and lectin binding.

Double positive cells for Dil-Ac-LDL uptake and lectin binding were analysed by fluorescence microscopy counting (*8*). An exact quantification of the double-positive cells is also commonly performed by FACS analysis (*26*).

Phenotypic analysis of total PBMCs and CD133$^+$ enriched cells from leukocyte-rich buffy coat showed two main homogeneous populations corresponding to the lymphocyte and monocyte gates (Fig. 2), both matching the previously described early EPC phenotype (*8*, *20*, *25*). In freshly isolated cells, as expected, a significant difference between the two compared methods was observed only in the highest percentage of cells expressing CD133 in lymphocyte gated population of CD133$^+$ enriched cells. Cells obtained by both methods coexpress endothelial and monocyte markers. Therefore, it is plausible that EPCs obtained by *in vitro* culture of total PBMCs or CD133$^+$ enriched cells are not only monocyte (*8*) but also lymphocyte derived from CD34$^+$ and CD133$^+$ haematopoietic cells, which can express some endothelial characteristic. During short-term culture, a significant decrease was observed only in percentage of cells expressing CD34 and CD133. The expression of VE-cad was maintained constant in EPCs from total PBMCs and CD133$^+$ enriched procedure (Fig. 3 and Table 1) consistently with the fact that EPCs maintain the progenitor cell characteristic during short-term culture. A significant increase in VE-cad expression, and other EC markers, i.e. KDR and von Willebrand factor, has been described during long-term culture of EPCs isolated from total PBMCs (*8*). However, although the surface markers expression allows the identification of cell type, their expression may change during culture in the presence of growth factors. This can explain why different groups have reported different surface markers profiles of EPCs (*8*, *14*). Thus, along with the surface markers expression, EPC characterization by functional test can be more appropriate.

To test the functional activity of EPCs from these two different isolation methods, we used TNF-α and glucose, known to impair EPC number via p38 mitogen-activated protein kinase phosphorylation (*25*). We found the inhibitory effect of TNF-α and glucose on EPC number isolated from total PBMCs was comparable to that already described (*25*). However, when CD133$^+$ enriched cells were used, the reduction of EPC number in response to TNF-α and glucose was more significant. Moreover, in accordance with the previous literature (*25*), an increased p38-phosphorylation was observed in TNF-α - and glucose- treated EPCs, but no significant differences were observed between EPCs obtained by procedures used in this study. Differences in the significance might be related to the method used for microscopy counts. Indeed, although the same number of experiments was performed for both microscopy counts and Western blotting, the number of fields and cells counted in each subfields ($n = 120$) were used to calculate statistical significance (Fig. 1). Several studies have shown the possible application of EPC as a therapeutic strategy for myocardial neovascularization but also for endothelial regeneration, in-stent restenosis (*2*, *3*). Recent small-scale trials have provided preliminary evidence of feasibility, safety, and efficacy in patients with myocardial and critical limb ischaemia (*2*).

Some studies have also shown that age and cardiovascular disease risk factors reduce the availability of EPCs and impair their function to various degrees (*25*, *26*). The relative scarcity of EPCs limits the ability to expand these cells in sufficient numbers for some therapeutic applications (*27–31*). In this context, the development of strategies to enhance the number and improve the function of circulating EPCs is still a priority.

When we compared the yield of Dil-Ac-LDL/Lectin double positive cells that can be obtained from both procedures starting from the same number of PBMCs, results indicated that in total PBMC preparation only ~10% of the cells attached to the plate, and ~23% of these cells were Dil-Ac-LDL/Lectin double positive cells, whereas following the $CD133^+$ enriched isolation procedure all seeded cells attached to the plate and ~50% of these cells were Dil-Ac-LDL/Lectin double positive cells. Thus, the yield of Dil-Ac-LDL/Lectin double positive cells among these two procedures is not particularly consistent (~2% from total PBMCs versus ~1% from $CD133^+$ enriched).

Previous studies demonstrate the applicability of $CD133^+$ selection with Miltenyi's immunomagnetic beads suggesting that depletion of T cells may be adequate for prevention of graft-vs-host disease (*28*, *29*). Infusion of $CD133^+$ positive stem cells to patients (from 2.6×10^4 to 1.1×10^5 cells/Kg) has been shown to be a useful method for safe transplantation with haploidentically mismatched stem cell allografts while avoiding lethal acute and chronic graft versus host disease (*29*). However, studies for a complete evaluation of the number of blood derived $CD133^+$ cells for clinical use are still lacking. Here we confirm that the $CD133^+$ enrichment allows the isolation of a homogenous progenitor population and, based reported studies (*2*, *29*), we estimated that the recovery of a consistent cell number may require ~75 ml of leucocyte-rich buffy coat (350–450 ml of peripheral blood are needed to obtain ~30 ml of leucocyte-rich buffy coat). Additional clinical trials are required to investigate the feasibility of this method to isolate $CD133^+$ cells sufficient to cure one patient.

The present study suggests that the two compared isolation procedures are equivalent since EPCs showed similar morphology and response to modulators of their functional activities. Despite such similarity the $CD133^+$ enrichment, although more expensive in terms of cost and time-consuming, allows the isolation of a progenitor population more suitable for supplying EPCs for clinical application. Indeed, some pilot clinical trials, still in progress, utilize EPCs for treatment of several diseases, such as myocardial infarction and chronic ischaemic cardiomyopathy (*2*, *27–31*).

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