

A comparison of CFU-GM, BFU-E and endothelial progenitor cells using *ex vivo* expansion of selected cord blood CD133⁺ and CD34⁺ cells

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Background

CD133 is a newly developed hematopoietic stem cell marker but little is known about its function. Whether CD133⁺ cell selection provides any advantage over CD34⁺ selection for hematopoietic stem cell isolation and transplantation is unclear. The present study compared colony formation and endothelial cell differentiation of these two cell types from umbilical cord blood (UCB).

Methods

Mononuclear cells from the same UCB samples were used for both CD133⁺ and CD34⁺ cell selection. Cells with 97.1% purity were incubated in semi-solid culture medium containing stem cell growth factor (SCGF) and G-CSF or erythropoietin (EPO). Purified cells were also cultured in M199 containing vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and insulin-like growth factor-1 (IGF-1).

Results

CD34⁺ and CD133⁺ cells produced similar numbers of CFU-GM colonies (median 43.25 and 30.5, respectively; $P > 0.2$). However, a

greater than four-fold difference in BFU-E colony formation was observed from CD34⁺ cells compared with CD133⁺ cells (median 35 and 8, respectively; $P < 0.04$). CD34⁺ cells gave rise to endothelial-like cells when stimulated with VEGF, bFGF and IGF-1. CD133⁺ cells were unable to produce this cell type under the same conditions.

Discussion

CD133⁺ cells produced smaller BFU-E colonies and were unable to differentiate into mature endothelial cells. CD34⁺ cells contained endothelial progenitors that could differentiate into mature cells of this lineage. Based on these data, it appears that CD133 offers no distinct advantage over CD34 as a selective marker for immunoaffinity-based isolation of hematopoietic stem cells and endothelial progenitor cells.

Keywords

CD133⁺ cells, CD34⁺ cells, endothelial progenitor cells, hematopoietic stem cells, umbilical cord blood.

Introduction

The efficient isolation of hematopoietic stem cell (HSC) is important for both clinical transplantation therapy and basic research. Expression of the CD34 cell-surface Ag has allowed identification of early hematopoietic stem and progenitor cells in BM, peripheral blood and umbilical cord blood (UCB). Over the last two decades, Ab to CD34 have been commonly used as a marker to select hematopoietic stem and progenitor cells [1]. The benefits of CD34⁺ cell selection are: (1) tumor or leukemic cells that

lack CD34 expression can be largely removed from potential transplant inocula; (2) mature T lymphocytes that are likely to give rise to GvHD can be depleted by several logs; and (3) the transplant inoculum can be debulked by removal of mature cells prior to *ex vivo* transduction for gene therapy applications [2]. However, several reports using mouse models have raised serious questions regarding CD34 expression by HSC [3,4]. In adult mice, Lin⁻ (myeloid/lymphoid/megakaryocytic/erythroid lineage-negative) CD34⁻ BM cells are regarded

as HSC because these cells can differentiate into multi-lineage colony-forming cells *in vitro* [5] and have long-term reconstituting ability after being transplanted into lethally irradiated mice [6]. Although there is abundant evidence of the existence of CD34⁺ HSC, the isolation and characterization of such cells from adult circulation has been hampered by the absence of specific markers. Whether more primitive stem cells exist that are resident in the CD34⁺ fraction needs further investigation.

HSC are also believed to be a source of endothelial progenitor cells [7–9]. During early development, hematopoiesis is closely associated with the formation of the vascular system. Targeted disruption of the gene encoding the vascular endothelial growth factor receptor-2 (VEGFR-2; also called Flk-1 and KDR in mice and humans) resulted in defects in both hematopoietic and endothelial cell development [10]. This suggested the existence of a ‘hemangioblast’ [11] that can give rise to both hematopoietic and endothelial cell lineages. Identification of hemangioblasts in the postnatal circulation has been hampered by the current lack of defined specific markers. A novel hematopoietic stem and progenitor cell marker AC133, recently designated CD133, has been identified and cloned [12]. In contrast to CD34, CD133 is not expressed on mature endothelial cells; whether it is an ideal marker for endothelial progenitor cell isolation is still under investigation.

The function of CD133 is so far unknown. The majority of cells reactive with CD133 Ab have been found to co-express CD34 [12–14]. Cells isolated by CD133 selection successfully engraft fetal sheep [14] and non-obese diabetic severe combined immunodeficiency (NOD/SCID) mice [15]. It is not clear whether use of this marker provides any distinct advantage over CD34 for HSC isolation and expansion, nor is it clear whether it is a marker for endothelial progenitor cells.

Human UCB is a rich source of HSC [16]. In contrast to adult BM-derived HSC, cord blood progenitors have distinctive proliferative advantages, including the capacity to form a greater number of colonies and a higher cell-cycle rate, as well as possessing longer telomeres [17–19]. In addition, cord blood can be obtained non-invasively, in contrast to invasive BM isolation. These properties favor the use of cord blood progenitors rather than adult peripheral blood and BM progenitors [20–22]. The aim of this study was to determine whether CD133⁺ cells are a

better choice than CD34⁺ cells for application in HSC and endothelial progenitor cell isolation and expansion.

Methods

Informed consent was obtained from expecting mothers prior to collection of CB. Local ethical committee provided approval for this study.

Cell purification by magnetic activated cell sorting

Mononuclear cells (MNC) were isolated from 40–50 mL of single-donor, fresh, UCB using Ficoll-Histopaque 1.077 (Sigma-Aldrich, Gillingham, UK) density centrifugation. After washing three times, twice with PBS containing 0.6% ACD-A and once with PBS/0.6%ACD-A plus 0.5% BSA, each sample of MNC was split into two parts and resuspended in PBS/ACD-A/BSA at 10⁸ cells/300 μ L.

For CD133 selection, one part of the MNC sample was subjected to immunomagnetic separation using a MACS isolation kit (Miltenyi Biotech, Surrey, UK), following the manufacturer’s recommendations. Briefly, MNC were incubated for 30 min at 6°C with FcR blocking reagent (human IgG) and CD133 microbeads (microbeads conjugated to monoclonal mouse anti-human CD133 Ab). After washing with pbs/ACD-A/bsa, the labeled cells were filtered through a 30- μ m nylon mesh and loaded onto a column surrounded by a magnetic field. CD133⁺ cells bound to the CD133 microbeads were eluted after the column was removed from the magnet. The collected cells were applied to a second column and the purification step was repeated. This isolation procedure has been reported previously [23]. The total number of CD133⁺ cells recovered was determined using a hemocytometer.

For CD34 selection, the second part of the MNC sample was subjected to immunomagnetic separation using a MACS isolation kit (Miltenyi Biotech), following the manufacturer’s recommendations. Briefly, MNC were incubated for 30 min at 6°C with mouse anti-human CD34⁺ and human IgG to prevent non-specific binding. Washing and column separation were performed in the same manner as for CD133⁺ cell selection, as described above.

Flow cytometry analysis

The expression of CD133 Ag on MNC derived from cord blood origin was estimated by three-color flow cytometry. Samples were labeled with FITC-, PE- and peridinin

chlorophyll protein (PerCP)- conjugated MAb [24]. Briefly, cells were incubated at 4°C with anti-CD45–PerCP (Becton Dickinson, Oxford, UK) for 20 min and with anti-CD34–FITC and anti-CD133–PE (Miltenyi Biotech) for further 10 min. The cells were then washed with PBS and analyzed on a FACScan flow cytometer (Becton Dickinson) using pre-stored settings. Data analysis was performed using Lysis II software (Becton Dickinson).

The purity of the CD133⁺ and CD34⁺ selected cells was determined by two-color flow cytometry. The CD133⁺ cells were labeled with anti-CD34–FITC and anti-CD133–PE-2 (Miltenyi Biotech); CD34⁺ cells were labeled with anti-CD45–FITC and anti-CD34–PE (Becton Dickinson). The cells were washed and analyzed as described above.

Adherent cells from both the CD34⁺ and CD133⁺ fraction were detached from the culture dish using trypsin at week 4. Cells were then washed and counted. Several endothelial cell-surface markers were evaluated by incubation for 30 min at room temperature with fluorochrome-conjugated MAb CD31 and CD105 (Serotec, Oxford, UK). The cells were then analyzed as described above.

CD133⁺ and CD34⁺ cell cultures

Liquid culture

CD133⁺ and CD34⁺ cells were cultured separately in fibronectin-coated six-well plastic dishes at $1-2 \times 10^5$ /mL containing M199 medium supplemented with 20% v/v FBS, vascular endothelial growth factor (VEGF; $1 \times$), basic fibroblast growth factor (bFGF; 1 ng/mL) and

insulin-like growth factor-1 (IGF-1; 2 ng/mL). All growth factors were purchased from Sigma -Aldrich. Cells were incubated for up to 4 weeks at 37°C in a humidified atmosphere containing 5% CO₂. Additional feeding was performed, depending on cell proliferation. Adherent cells from both CD133⁺ and CD34⁺ cultures were used for subsequent analysis.

Semi-solid culture

CD133⁺ and CD34⁺ cells were resuspended in IMDM containing 2% v/v FBS at a concentration of 5×10^3 cells/mL, and mixed with four volumes of methylcellulose-based semi-solid culture medium (MethoCult™ H4230; StemCell Technologies, London, UK), containing stem cell growth factor (SCGF; 20 ng/mL) and G-CSF (6000 U/mL) for generating CFU-GM, and SCGF (20 ng/mL) and erythropoietin (EPO; 1.5 U/mL) for BFU-E (all from Tebu-bio, Peterborough, UK). Aliquots of the mixtures (1 mL/10³ cells) were plated in 35-mm² grid dishes and incubated for 14 days at 37°C in a humidified atmosphere with 5% CO₂ at 37°C. The growth factor combination and culture conditions used have been reported previously [25]. Each experiment was carried out in duplicate.

Cell immunolabeling with von Willebrand factor

After 4 weeks in liquid culture, cells were incubated in a blocking buffer consisting of PBS/BSA, for 30 min at 37°C, and then incubated with anti-von Willebrand factor (vWF) (rabbit anti human vWF; Sigma-Aldrich) at a dilution of 1:100 (v/v in PBS) for 30 min at 37°C. Excess Ab was

Table 1. Sequence of RT-PCR primers

Target gene	Sequences (5'–3')	Size (bp)
KDR	CTGTAACAGATGAGATGCTCCAAGG (S) ACCAAAGGGGCACGATTCGGTC (AS)	209
eNOS	CCAGCTAGCCAAAGTCAACAT (S) GTCTCGGAGCCATACAGGATT (AS)	354
VE-cadherin	CCAGTGAGACAAAAACGGG (S) GCATCACCAAGTGAGCAGAA (AS)	226
PECAM-1	TGTTGACATGAAGAGCCTGC (S) ACAGTTGACCCTCACGATCC (AS)	349
GAPDH	CTACTGGCGCTGCCAAGGCTGT (S) GCCATGAGGGTCCACCACCCTGT (AS)	358
vWF	GTTCGTCCTGGAAGGATCGG (S) CACTGACACCTGAGTGAGAC (AS)	696

Table 2. Percentage of CD133⁺ cell in MNC from 14 UCB samples

Phenotype	Median (%)	Range (%)
CD133 ⁺	0.665	0.06–2.15
CD133 ⁺ CD34 ⁺	0.35 (72.16)	0.02–1.23
CD133 ⁺ CD34 ⁻	0.135 (28.45)	0.03–0.92
CD133 ⁺ cells are considered equal to 100%		

removed by washing with PBS, and the cells were then re-incubated for another 30 min at 37°C with a secondary Ab (FITC-conjugated goat anti-rabbit IgG; Sigma-Aldrich) at a 1:160 dilution (v/v in PBS). After further washing with PBS, the cells were examined by confocal microscopy (Leica TCS SP2 UV, Heidelberg, Germany, confocal system).

RNA extraction and RT-PCR

Poly(A) RNA was extracted from freshly isolated CD34⁺ and CD133⁺ cells or BFU-E or CFU-GM colonies using Dynabeads® mRNA DIRECT™ Kit* (DynaL Biotech, Liverpool, UK), following the manufacturer's protocols. Briefly, colonies from BFU-E and CFU-GM were visualized with the aid of an inverted microscope and plucked from serum-free cultures on day 14 using a Pasteur pipette. Randomly selected colonies (up to 20 colonies) from each culture dish were utilized for experiments. Colonies were resuspended in 15 mL IMDM and incubated for 30 min at 37°C to dissolve the methylcellulose. The cells were washed twice in PBS prior to mRNA extraction. RT-PCR was performed using an Access RT-PCR system according to the manufacturer's instructions (Promega, Southampton, UK). mRNA was reversibly transcribed to

cDNA at 48°C for 45 min, followed by 94°C for 2 min. Amplification reactions were carried out for 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 68°C for 1 min, followed by a final extension at 68°C for 7 min. The PCR products were detected on a 1.5% agarose gel with ethidium bromide. All primers used in this experiment are shown in Table 1. All experiments were repeated at least three times, and only results that were consistently reproducible are reported. All RT-PCR experiments were carried out under identical conditions.

Statistical evaluation

The Wilcoxon signed rank test was applied to statistical analyzes of differences between CD133⁺ and CD34⁺ forming BFU-E or CFU-GM.

Results

Flow cytometry analysis of expression of CD133 on UCB MNC

The gating strategy and analysis of the cells expressing either CD34 or CD133 were carried out according to the protocol recommended by the International Society for Hematotherapy and Graft Engineering (ISHAGE) for CD34⁺ cell determination by flow cytometry [26]. The percentage of CD133⁺, CD133⁺/CD34⁺, and CD133⁺/CD34⁻ cells observed in MNC is reported in Table 2. The percentage of CD133⁺ cells in the samples ranged from 0.06% to 2.15%, with a median of 0.665%. Within the CD133⁺ cell population, a high percentage of cells (72.16% median value) co-expressed CD133 and CD34 Ag, whereas 28.45% (median value) of cells expressed CD133 but not CD34 Ag (Table 2).

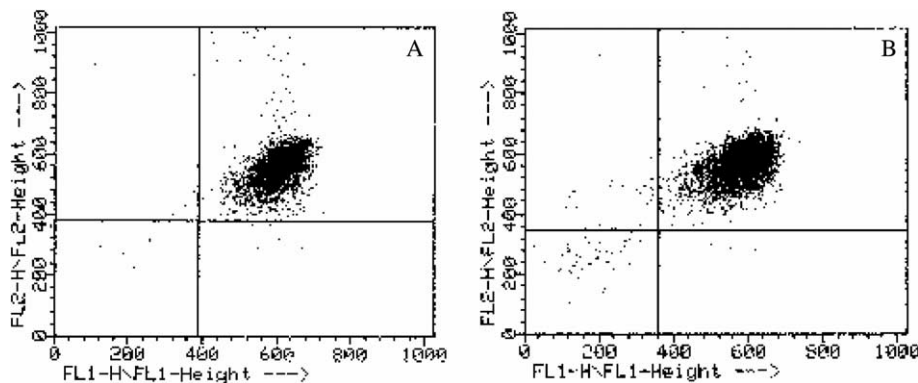


Figure 1. A representative flow cytometry profile of isolated human cord blood CD133⁺ and CD34⁺ cells. (A) CD133⁺ cells labeled with CD45-FITC and CD133-PE. (B) CD34⁺ cells labeled with CD45-FITC and CD34-PE.

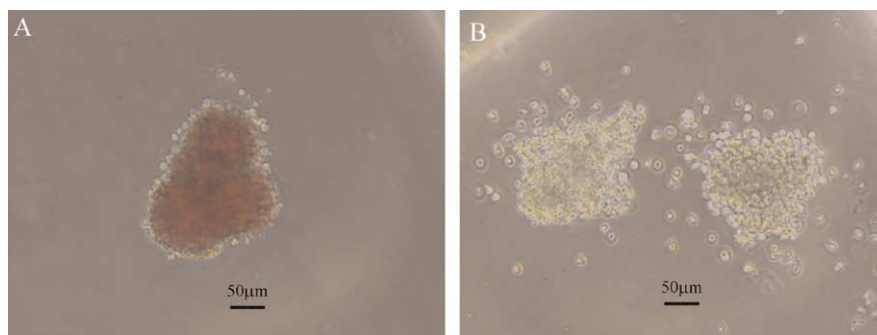


Figure 2. Morphology of colonies generated from $CD133^+$ cells. (A) $CD133^+$ cells cultured in methylcellulose-based semi-solid medium supplemented with SCGF and EPO to form CFU-E and BFU-E. (B) $CD133^+$ cells in methylcellulose-based semi-solid medium with SCGF and G-CSF to form CFU-GM.

Purity of $CD133^+$ and $CD34^+$ isolates

As determined by flow cytometry, the median purity of the positively selected $CD133^+$ and $CD34^+$ cells after magnetic cell sorting was 97.1% (range 91.99–99.2%) and 95.1% (range 89.3–98%) of the total cells, respectively. The majority (95%) of $CD133^+$ isolates were also $CD34^+$ (Figure 1). Fewer $CD34^+$ separated cells were also $CD133^+$ (mean 72%; data not shown).

CFU-GM and BFU-E content in UCB $CD133^+$ and $CD34^+$ cells

CFU-GM and BFU-E formation was used to compare the colony-forming capacities of $CD133^+$ and $CD34^+$ cells derived from the same CB MNC. In six separate experiments, 10^3 cells/mL were cultured for 14 days in methylcellulose-based semi-solid medium containing different hemopoietic growth factor (HGF) combinations (Figure 2). $CD133^+$ and $CD34^+$ cells displayed no significant differences in CFU-GM formation (median number of colonies 43.25, cf. 30.5, $P > 0.2$). In contrast, a significant difference in BFU-E generation of these cells was observed when cultured in medium supplemented with SCGF + EPO. A greater than four-fold difference in colony-forming capacity was observed for $CD34^+$ cells under these later conditions (median number of colonies 35, cf. 8, $P < 0.04$) (Figure 3).

Endothelial cell differentiation

Freshly isolated $CD34^+$ and $CD133^+$ cells were grown on plastic fibronectin-coated six-well plates at 2×10^5 cell/mL in the presence of VEGF, bFGF and IGF-1. $CD34^+$ cells appeared spindle shaped by days 4–5. In contrast, $CD133^+$ cells appeared spindle shaped by days 5–7. After 3–4 weeks, $CD34^+$ cultures became confluent and

consisted of a monolayer of spindle-shaped cells, with some areas having typical ‘cobblestone’ morphology (Figure 4A); the cultures stained positively for vWF (Figure 4B) and FACS analysis further confirmed expression of CD31 and CD105 (Figure 4C). $CD133^+$ cultures remained spindle shaped at the same stage (Figure 5A) and stained negatively for vWF (Figure 5B), while CD31 and CD105 were undetectable in these cells by FACS (data not shown). Cultures of $CD133^+$ cells maintained for 6–8 weeks retained the same morphology and negatively stained for vWF (data not shown).

RT-PCR analysis

Both $CD133^+$ and $CD34^+$ cells expressed a number of endothelial cell markers, including vWF, KDR, VE-cadherin and PECAM-1 mRNA, but freshly isolated $CD133^+$ cells did not express eNOS mRNA (Figure 6).

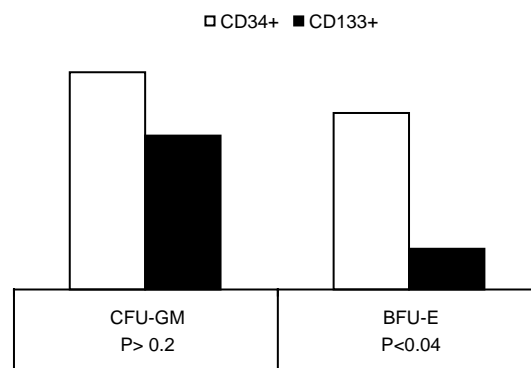


Figure 3. Comparison of the colony-forming capacities of $CD133^+$ and $CD34^+$ cells isolated from same UCB. The median number of BFU-E colonies was 35 from the $CD34^+$ fraction and 8 from the $CD133^+$ fraction ($P < 0.04$). The median of CFU-GM derived from the $CD34^+$ fraction was 43.25 and that from $CD133^+$ was 30.5 ($P > 0.2$).

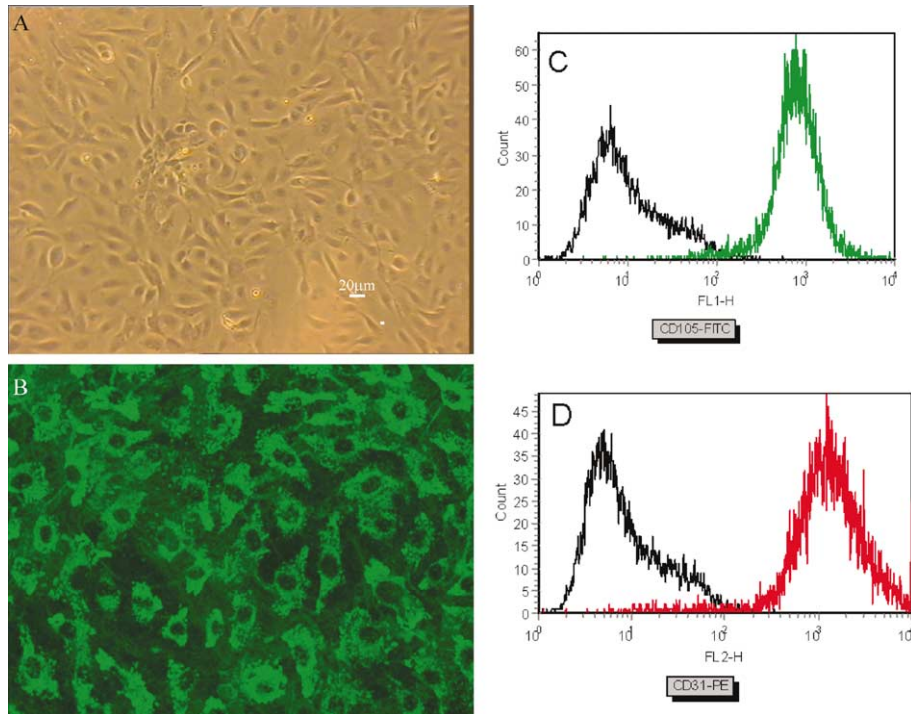


Figure 4. Morphology of cells differentiation from $CD34^+$ in M199 medium supplemented with VEGF, bFGF and IGF-1. (A) Phase-contrast micrograph of cells from the $CD34^+$ fraction with cobblestone morphology at week 4; these cells stained positively with vWF (B). FACS analysis of cells from $CD34^+$ cell cultures at week 4; these cells showed expression of endothelial cell markers CD105 (C) and CD31 (D).

The function of CD133 has not yet been clarified, partly because the putative ligand of CD133 protein has not yet been identified [9,12]. To learn more about the biologic role of CD133 in hematopoiesis and vasculogenesis, we analyzed the expression of the CD133 gene by RT-PCR. As shown in Figure 7, CD133 mRNA was not detected in CFU-GM, BFU-E and fibroblast-like cells that were potentially endothelial progenitor cells. Each experiment was performed three times.

Discussion

CD133 is a newly described marker of early human hematopoietic cells [12,14]. The role of CD133 in hematopoiesis and vasculogenesis is uncertain. In the present study, we compared the colony-forming capacity and gene expression of $CD133^+$ vs. $CD34^+$ cells isolated from UCB MNC.

FACS analysis showed that, in our 14 UCB samples, the median number of $CD133^+$ cells was 0.665%

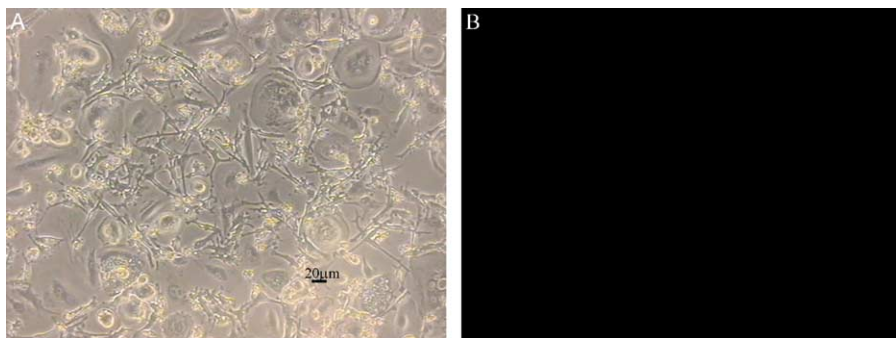


Figure 5. Morphology of cells differentiation from $CD133^+$ in M199 medium supplemented with VEGF, bFGF and IGF-1. (A) Phase-contrast micrograph of cells from $CD133^+$ cells at week 4, and negatively stained with vWF (B). CD31, CD105 and KDR expression was not detected by FACS analysis (data not show).

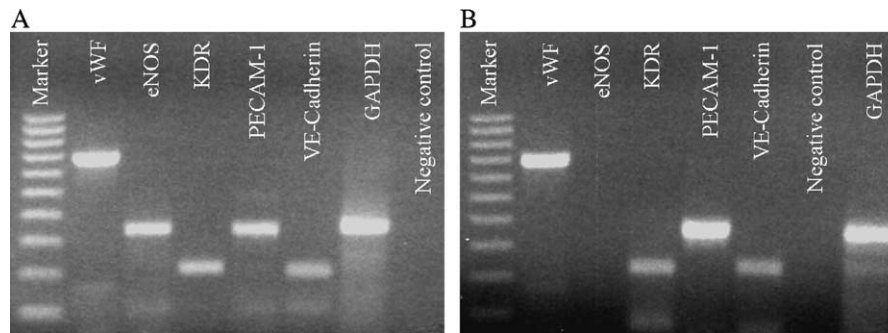


Figure 6. RT-PCR analysis of endothelial gene expression on CD133⁺ and CD34⁺ cells. (A) Freshly isolated CD34 cells express a number of endothelial markers, including vWF, eNOS, KDR and PECAM-1. (B) No eNOS mRNA express on freshly isolated CD133 cells. GAPDH, control of mRNA integrity; negative control, dd-water.

(range 0.06–2.15% of total MNC), which was lower than that reported by others [24]. The lower percentage of CD133⁺ cells observed could reflect the different staining methods applied. In this study, three-color immunofluorescence staining was used, which is reported to be more accurate than one-color staining [27]; 72.16% of the human UCB CD133⁺ cells co-expressed CD34 Ag, while the other 28.45% CD133⁺ cells were CD34⁻. This is consistent with other reports in the literature [28].

Our study established that CD133⁺ and CD34⁺ cells differed in their colony-forming capacity. The CD34⁺ fraction produced more colonies than CD133⁺ cells under the defined culture conditions. The median number of CFU-GM colonies derived from CD34⁺ cells was 43.25, compared with 30.5 from CD133⁺ cells. However, this difference was not significant ($P > 0.1$). In contrast, the median of BFU-E from the CD34⁺ fraction was 35, compared with 8 observed from the CD133⁺ fraction, and the difference was statistically significant ($P < 0.04$). The

total number of colonies generated from CD34⁺ cells was 78.25 (median), while that derived from CD133⁺ cells was 38.5 (median); this difference was also significant ($P < 0.05$). The observation that the colony-forming capacity of CD133⁺ and CD34⁺ cells from the same sample was significantly different could be explained by the fact that there were non-hematopoietic cells in the CD133 fraction, such as epithelial cells, trophoblasts and other unidentified cells [29,30]. Similar findings have been reported, although the HSC were isolated from mobilized peripheral blood other than UCB [31]. The CD133⁺ fraction in general produced smaller colonies under the same culture conditions (initial cell number, cytokines, etc.). A potential reason for this observation is that lineage-committed progenitors may not express the CD133 Ag because expression is rapidly down-regulated when the cells proliferate and differentiate [12,14]. In our study, CD133 mRNA expression was also lost in cells isolated from CFU-GM and BFU-E (Figure 7), which substantiates these findings.

It has been discussed for a long time whether hematopoietic and endothelial lineages arise from a common precursor, the hemangioblast [32,33]. In a recent study, Choi *et al.* [11] identified the hemangioblast within a murine embryonic stem cell-derived precursor population. Although not proven, it can be hypothesized that this finding in general is transferable to the human system. Hence it may no longer be questionable whether the hemangioblast exists, but rather whether it can persist into adult life. Several groups have now identified endothelial progenitor cells (EPC) resident in BM, peripheral blood and UCB. Furthermore, these cells can be driven into the endothelial development pathway [7,9,34]. Investigators from these groups employed anti-CD34 Ab, separated a

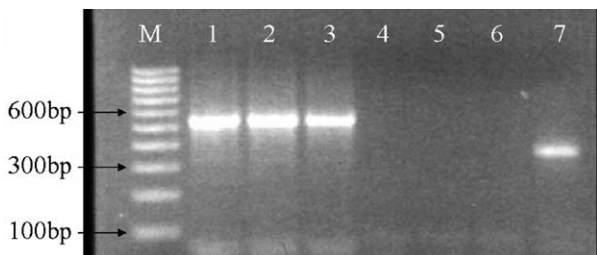


Figure 7. RT-PCR analysis of CD133 expression; CD133 mRNA (561 bp) can be detected in freshly isolated CD133, CD34 and CD34⁻ depleted MNC but rapidly down-regulate after differentiation. M, PCR marker; 1, CD133⁺ cells; 2, CD34⁺ cells; 3, CD34⁻ MNC; 4, CFU-GM; 5, BFU-E; 6, fibroblast-like cells; 7, GAPDH (358 bp, control of mRNA integrity). The experiment was repeated three times with the same results.

group of CD34⁺ cells and induced these cells to differentiate into mature endothelial cells. As anti-CD34 Ab bind to mature endothelial cells, it is ideal to have another HSC marker available. In contrast with CD34, CD133 Ag, which is not expressed on mature endothelial cells, may provide such a marker. However, our results do not support this concept. We confirmed that cells from the CD34⁺ fraction differentiated into endothelial cells, as judged by morphology and positive staining with vWF, while cells from the CD133 fraction failed to show similar patterns of growth under the same culture conditions. Our results suggest that CD133 Ag is not expressed on EPC or, alternatively, that some as yet unidentified growth factor, able to promote more primitive cell differentiation, may be absent in our culture conditions. We found that CD133 mRNA expression was down-regulated during differentiation (Figure 7). If CD133 is an early marker for HSC, but the expression is rapidly lost during differentiation [12,14], committed progenitors such as myeloid precursors, lymphoid precursors and endothelial precursors may not express CD133. If this is indeed the case, using CD133 as a marker and tool for HSC isolation could inevitably mean that some precursors would be excluded and lost during any isolation procedure.

In conclusion, CD133 is expressed on human UCB HSC and its expression is down-regulated during differentiation. Furthermore, CD133⁺ cells produced smaller BFU-E colonies, lacked eNOS expression and were unable to differentiate into mature endothelial cells. Based on these data, it appears that CD133 offers no distinct advantage over CD34 as a selective marker for immunoaffinity-based isolation of HSC and endothelial progenitor cells.

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