Flow Cytometric Immunophenotyping of Umbilical Cord and Peripheral Blood Haematopoietic Progenitor Cells by Different CD34 Epitopes, CD133, P-Glycoprotein Expression and Rhodamine-123 Efflux

Manja Kamprad^a Sven Kindler^b Nicole Schuetze^c Frank Emmrich^a

^a Institut für Klinische Immunologie und Transfusionsmedizin,

^b Einheit für multidisziplinäre Intensivmedizin, Universitätsklinikum Leipzig,

^c Institut für Immunologie, Veterinärmedizinische Fakultät, Universität Leipzig, Germany

Key Words

Haematopoietic stem cell · CD34 epitopes · CD133 · Rhodamine-123 · P-glycoprotein · Umbilical cord blood

Summary

The flow cytometric enumeration of CD34+ cells, comprising both haematopoietic stem (HSC) and progenitor cells (HPC) is currently widely used to assess suitability of stem cell transplants for transplantation. However, to ensure the long-term reconstruction in human recipients, the enumeration specifically of those HSC possessing self-renewing and differentiation potential would be preferable. In the present study we addressed a multiparametric flow cytometric profiling of haematopoietic stem cells of umbilical cord (CB) and peripheral blood (PB) using stem cells markers (epitope-specific CD34, CD133, rhodamine-123 (Rho123) efflux, p-glycoprotein) to assess their stem cell features. In contrast to CB, the CD34 class III compartment of PB did not include all CD34 class I- and class II-expressing cells. In CB and PB, there was no clear correlation between CD34 epitope and CD133 expression. Furthermore, we detected CD133+ CD34- cells (0.07% of mononuclear cells) in CB. The functional ability to exclude Rho123 was mainly restricted to CD34- and CD133-co-expressing cells. However, CB cells showed a higher activity. Functionally active cells were characterised by their expression of the verapamil-sensitive protein pump p-glycoprotein 170 using the monoclonal antibody MRK16. It is therefore possible to use multiparametric flow cytometry to estimate the numbers of immature stem cells as CD34+ CD133+ (Rho^{dull}) MRK16+. This would allow a more precise assessment of transplant quality, especially with respect to their long-term engraftment potential, and supports haemotherapy of malignant diseases.

Schlüsselwörter

Hämatopoetische Stammzelle · CD34-Epitope · CD133 · Rhodamin-123 · P-Glykoprotein · Nabelschnurblut

Zusammenfassung

Die durchflusszytometrische Bestimmung des Gehalts an CD34+ Zellen, die sowohl hämatopoetische Stamm- als auch reifere Progenitorzellen einschließen, wird bis jetzt häufig genutzt, um die Eignung eines Stammzelltransplantats für die Transplantation zu beurteilen. Um eine dauerhafte Blutbildung im Empfänger zu garantieren, sollten jedoch jene hämatopoetische Stammzellen bestimmt werden, welche die Fähigkeit der Selbsterneuerung und der Differenzierung aufweisen. Ziel dieser Studie war es daher, mittels verschiedener Stammzellmarker, wie CD34-Epitope I–III, CD133, Rhodamin-123(Rho123)-Efflux und P-Glykoprotein 170, Subtypen von hämatopoetischen Stammzellen mit hohem Stammzellpotential im Nabelschnurblut und peripheren Blut zu benennen. Im Gegensatz zu Nabelschnurblut umfasst das CD34-Klasse-III-Zellkompartiment des peripheren Blutes nicht alle CD34-Klasse-I- und -Klasse-II-tragenden Zellen. Weder im Nabelschnurblut noch im peripheren Blut gab es eine klare Korrelation zwischen der CD34-Epitop- und der CD133-Expression. Im Nabelschnurblut sind ungefähr 0,07% aller mononukleären Zellen CD133+ CD34-. Die Fähigkeit zum Rho123-Efflux kennzeichnet im Wesentlichen CD133+ CD34+ Zellen. Die Zellen des Nabelschnurblutes zeigen hierbei eine höhere Aktivität. Funktionell aktive Zellen lassen sich durch die Expression der Verapamil-sensitiven Proteinpumpe P-Glykoprotein mittels des monoklonalen Antikörpers MRK16 darstellen. Somit lässt sich mit Hilfe der multiparametrischen durchflusszytometrischen Analyse ein unreifes Stammzellkompartiment als CD34+ CD133+ (Rho^{dull}) MRK16+ darstellen. Die Bestimmung dieser Zellpopulation würde eine präzisere Bewertung der Transplantatqualität erlauben, besonders im Hinblick auf die Sicherung einer dauerhaften Blutbildung im Empfänger nach einer Hämotherapie mit adulten Stammzellen.

Introduction

Stem cell transplantation is an established procedure to treat haematological malignancies by restoring a functional blood system. The long-term outcome of recipients depends on the application of sufficient amounts of haematopoietic stem cells (HSC) possessing self-renewing and differentiation potential [1], an issue of particular relevance when the available graft material is small, as is often the case in umbilical cord blood. The flow cytometric identification and quantification of cells representing the most immature progenitors in a transplant unit would allow a more precise definition of the engraftment potential [2]. Multiplexed and high-content analysis [3–5] of the subsets of progenitor and stem cells should further support their unequivocal identification and understanding of their functions.

Despite current uncertainty regarding its function, the CD34 antigen remains the most widely used marker to define haematopoietic stem/progenitor cells (HSC/HPC). The reconstruction of an adaptive immune system in human recipients after transplantation of enriched CD34+ cells clearly demonstrates the stem cell potential within this population. However, studies on short- and long-term recovery after application of isolated CD34+ cells in immunodeficient mice have shown that the number of CD34-expressing cells is not necessarily a good indicator of self-renewal and differentiation capacity. The CD34+ compartment does include quiescent cells with true stem cell properties, including self-renewal capability, but also encompasses later cells at various stages of differentiation (lineage-committed cells, maturing precursors lacking self-renewing ability). Previous studies indicate that the functional characteristics of HPC and of leukaemic blast cells of acute myeloid leukaemia can be related to their CD34 epitope class expression [6–8]. CD34 is a heavily glycosylated transmembrane sialomucin (105–120 kDa). The polymorphism of CD34 glycosylation and therefore the specificity of monoclonal antibodies (mAbs) assessed by their differential susceptibility to enzymes, tissue reactivity and Western blotting analysis is the basis for extended classification of the three main epitopes [9, 10].

The stem cell marker CD133 [11] has been found on HSC, embryonic stem cells and tissue-specific adult stem cells as well as on some malignant cells [12–14]. Several studies suggest that CD133 provides a promising selection marker for a highly non-committed subset of HPC possessing self-renewing and differentiation ability as well as high clonogenic capacity [15–17]. The majority of CD133-expressing cells of different stem cell sources can be found in the CD34 compartment [18, 19].

Primitive haematopoietic cells are clearly defined by their verapamil-sensitive ability to excrete rhodamine-123 (Rho123) [20–22]. P-glycoprotein 170 (Pgp-170), breast cancer resistance protein (BCRP1) as well as mitoxantrone-resistance protein (MRX1) are predominantly responsible for this activ-

ity of uncommitted HSC. All these proteins are members of the ATP-binding cassette (ABC) transporter family. These transporters are expressed differentially during haematopoiesis, with the highest level in primitive bone marrow stem populations that are CD34+^{low} or CD34– [23]. The expression of the *mdr1a* gene encoding the Pgp-170 pump is highest in long-term re-populating cells and progressively decreases with maturation [24]. Furthermore, it has been demonstrated that age-related alterations in both phenotype and function of haematopoietic compartment in mice correlated to up-regulated activity of multi-drug resistance (MDR) proteins in HSC from older animals [22, 25].

The aim of this study was to phenotypically characterise by multiparametric flow cytometry a HPC subpopulation from umbilical cord blood representing the most immature compartment. For this reason, CD34 epitope mapping in relation to CD133 and Pgp-170 expression and their functional capacity to exclude Rho123 were measured.

Materials and Methods

Cells

Cord blood was collected following normal, full-term pregnancies and deliveries with informed consent from the mother. The local ethical commission accepted the protocol. The cord blood was obtained by puncture of the cord blood vein and stored in EDTA monovettes (Sarstedt, Nümbrecht, Germany). All samples were processed within 24 h of collection. Human peripheral blood cells were prepared from buffy coat (adults) or citrate-conserved blood (children).

Briefly, the mononuclear cell fraction was isolated by Ficoll (Pharmacia Biotech, Freiburg i.Br., Germany) gradient separation followed by ammonium chloride / EDTA lysis of erythrocytes. Afterwards the cells were washed with phosphate-buffered saline (PBS) / 1% foetal calf serum (FCS).

Immunophenotyping by Flow Cytometry Analysis

The following antibodies were used for cell staining: CD34 type III (clone 581, phycoerythrin(PE)- or phycoerythrin-cyanin-5(PC5)-labelled; Immunotech, Beckman Coulter, Krefeld, Germany), CD34 type II (QBEnd10, PC5-labelled; Immunotech, Beckman Coulter), CD34 type I (Immu133.3, PE- or PC5-labelled; Immunotech, Beckman Coulter), CD34 type I and II (Immu409, QBEnd10, PE-labelled; Immunotech, Beckman Coulter), CD133-PE (AC133/1; Miltenyi Biotec, Bergisch Gladbach, Germany), CD45-APC (clone J33; Immunotech, Beckman Coulter), Pgp-170 (MRK16, 4E3-biotin, UIC2; Hölzel Diagnostika GmbH, Köln, Germany), goat-anti-mouse-PE (Hölzel Diagnostika GmbH), goat-anti-mouse-FITC (Dako, Hamburg, Germany), goat-anti-mouse-Alexa Fluor 594 (Caltag/Invitrogen, Karlsruhe, Germany), Streptavidin-FITC (Hölzel Diagnostika GmbH), Streptavidin-Quantum-Red (Sigma, Deisenhofen, Germany), mouse IgG-PE/PC5/APC (Immunotech, Beckman Coulter) and non-conjugated mouse IgG (BD Biosciences, Heidelberg, Germany). The co-expression pattern of different CD34 epitopes was analysed by combination of PC5- and PE-conjugated CD34 epitope-specific antibodies, and CD45-APC. The estimation of CD133-expressing cells was done by CD133-PE, PC5-conjugated CD34 epitope-specific antibody, and CD45-APC. The quantification of Pgp-170-expressing cells was performed by MRK16/goat-anti-mouse-FITC or 4E3-biotin/streptavidin-FITC in combination with PC5-labelled CD34 epitope-specific antibody or CD133-PE, and CD45-APC. Generally, preliminary results showed

Fig. 1. Flow cytometric analysis of CD34 epitope-expressing cells of cord blood (**a, b**) and peripheral blood (**c**).

a, b Typical example of the characterisation of CD34 epitope-expressing cells. Mononuclear cord blood cells were labelled with CD34 epitope-specific antibodies. Dot plots show typical staining of CD34^I-expressing cells (**a**) or CD34^{II}-expressing cells (**b**) in relation to CD34^{III} expression (gated on CD45+ cells with low side scatter properties, not shown). **c** Mononuclear cells (n = 6) were stained with an antibody cocktail of CD34^I-CD34^{II}-specific antibodies (Immu409, QBend10) labelled with PE and PC5-labelled CD34^{III} antibody. The localisation of subpopulations in relation to their CD34^{III} expression was verified using singular CD34^I- (Immu133.3, n = 4) and CD34^{II}-specific antibody (QBend10, n = 8). Generally, all samples were stained with CD45-APC. To enumerate HPC, the ISHAGE gating strategy was used.

that the estimation of cell subsets was independent of the fluorochrome with which the monoclonal antibody was labelled. Labelling of 5×10^5 cells was carried out using mAbs in 100 µl PBS / 1% FCS at room temperature for 20 min. After washing twice with PBS / 1% FCS (250 × g, 5 min), cells were fixed with 3% formaldehyde. Isotype-identical mAbs served as controls. The analysis was performed on a FAC-SCalibur (BD Biosciences) using CellQuest software (BD Biosciences). Generally, the estimation of HPC and their subpopulations were based on the ISHAGE (International Society of Hematotherapy and Graft Engineering) guidelines [26]. Only CD45+ cells were used for further analysis.

Rho123 Efflux

Rho123 is a vital dye for mitochondria with an emission maximum at 533 nm (excitation by argon laser 488 nm). For labelling, 5×10^5 cells in 100 µl PBS / 1% FCS were incubated with Rho123 0.86 µg/ml (Invitrogen, Molecular Probes, Karlsruhe, Germany) at 37 °C for 30 min in the dark. After washing twice with cold PBS / 1% FCS, one sample was incubated in 2 ml Hanks-buffered saline at 37 °C for 40 min to evaluate the functional capacity of cells excluding Rho123. This reaction was stopped by addition of 2 ml cold PBS / 1% FCS. A control sample was stored at 4 °C for 40 min. After washing twice with cold PBS / 1% FCS, samples were stained for immunophenotyping with CD34 class I (CD34^I), class II (CD34^{II}) or class III (CD34^{III}) specific PC5-labelled antibody in combination with CD133-PE and CD45-APC. To prove the correlation between Rho123 efflux and Pgp-170 expression, cells were labelled with Pgp-170-specific antibody MRK16/goat-anti-mouse-Alexa Fluor 594 or 4E3-biotin/streptavidin-Quantum Red in combination with CD34^{III}-PE or CD133-PE, and CD45-APC. All labelling and washing steps were done by 4 °C.

The specificity of transmembrane-bound protein pump was investigated by block experiments with verapamil (Sigma). After labelling with Rho123, cells were incubated with 25 µmol/l verapamil solution (PBS / 1% FCS) at 37 °C for 40 min.

Statistical Analysis

To obtain statistically valid values of the relative distribution of HPC and their subpopulations in samples, we acquired at least 50,000 and 100,000 CD45+ cells of cord blood and peripheral blood samples, respectively. All results were expressed as the mean of data obtained from six or more (up to 45) separate experiments ± 1 standard deviation. Significance levels were determined using a two-sided Student's t-test or Kruskal-Wallis-test.

Results

CD34 Epitope Expression Analysis on Mononuclear Cells of Cord Blood and Peripheral Blood

The CD34 antigen, the most widely used marker for detection and enrichment of HSC/HPC, describes a very heterogeneous cell fraction in terms of self-renewal capacity and lineage commitment. Firstly, we analysed the expression pattern of the CD34 epitopes on cord blood and peripheral blood with antibodies against CD34^I, CD34^{II} and CD34^{III} separately. ISHAGE guidelines were used to quantify the expression patterns of surface molecules on HSC, the percentage of specific

Fig. 2. Flow cytometric analysis of expression of CD133 and different CD34 epitope classes and percentage of CD133+ CD34[−] fraction of CD45^{dim/+} mononuclear cells in cord blood samples (CD34^{III} n = 15, CD34^{I/II} n = 8). Estimation of HPC subsets based on their CD45 expression and morphological properties (ISHAGE gating strategy). **a** The dot plot shows exemplarily the CD133 expression in relation to CD34^{III} expression of cord blood cells. **b** The percentage of the CD133+ CD34[−] fraction of CD45^{dim/+} cord blood mononuclear cells is given as box plot (box plot: median value = horizontal line in the box, 25/75 percentile = box borders; 10/90 percentile = error bars).

cell population being expressed relative to mononuclear cells with diminished/positive expression of CD45 and low side and forward scatter properties.

The majority of cord blood HPC express the epitope CD34^{III} ($0.71 \pm 0.56\%$ of mononuclear cells; $n = 15$), followed by CD34^I ($0.61 \pm 0.33\%$) and CD34^{II} ($0.55 \pm 0.40\%$). Analysis of co-expression with CD34 epitope-specific antibodies showed that the CD34^{III} epitope fraction included also all CD34^I and CD34^{II} epitope-bearing cells (fig. 1a, b).

In peripheral blood (CD34^{I/II/III} $0.27 \pm 0.20\%$; $n = 45$) the most prominent epitope was again found to be CD34^{III} (89.9% of CD34^{I/II/III}, $0.26 \pm 0.18\%$), followed by CD34^{II} (58.1% of CD34^{I/II/III}, $0.16 \pm 0.14\%$) and CD34^I (40.3% of CD34^{I/II/III}, $0.10 \pm 0.08\%$). In contrast to cord blood, the co-expression of CD34^I with CD34^{III} is a very rare event (approximately 2.3% of CD34⁺ cells). Moreover, nearly 50% of peripheral blood CD34^{II}-expressing cells could not be stained by CD34^{III}-specific antibody (data not shown). As shown in figure 1c, these CD34^{I/II}+ CD34^{III}− cells clearly appeared as a distinct subpopulation. The majority of CD34^I- and half of CD34^{II}-expressing cells in peripheral blood were not detected by typically used CD34^{III} antibodies.

Correlation of CD133 Expression with Specific CD34 Epitopes

We investigated if the expression of CD133 as a marker describing a primitive haematopoietic cell subpopulation associated with self-renewal, high clonogenic proliferation and differentiation capacity correlates with specific CD34 epitope expression on cord blood cells (fig. 2a). However, no significant differences with respect to the expression of CD133 and different CD34 epitopes in cord blood were found. Approximately 60% of CD34^I-, CD34^{II}- and CD34^{III}-expressing mononuclear cells carried CD133. In peripheral blood only a minor population of CD34^{I/II/III}-expressing cells were positive for CD133 ($15.0 \pm 4.1\%$).

Generally, $0.50 \pm 0.32\%$ of cord blood mononuclear cells ($n = 28$) and $0.06 \pm 0.04\%$ of peripheral blood mononuclear cells

could be stained with CD133 antibody. Further, a small population of CD133+ cells (0.07 ± 0.07 of mononuclear cells) exists in umbilical cord blood, which is not associated with any CD34 epitope expression (fig. 2b). High individual variances were noticed. Additionally, we analysed the CD34 epitope expression pattern within the CD133 populations. CD133+ cord blood cells express predominantly CD34^{III} or CD34^{II} (approximately 85%) compared to CD34^I (approximately 70%). In peripheral blood, the identification of CD133+ CD34[−] cells as a very rare event was depending on the CD34 antibody used. Using an antibody mixture including CD34^{I/II/III} epitopes we found a rare frequency of CD133+ CD34[−] cells (1/8), in contrast to single CD34^{III}-specific antibody (3/8) – here, 75, 60 and 30% of CD133+ cells were CD34^{III}−.

Rho123 Efflux Activity of Cord Blood and Peripheral Blood Progenitor Cells

The functional capacity of HPC to pump out the mitochondrial dye Rho123 characterises a subpopulation associated with high re-population and long-term engraftment ability. We examined whether or not this functional activity depends on expression of different CD34 epitopes or CD133 (fig. 3a, b). In cord blood, independently of the tested stem cell marker, the relative number of Rho123^{low} cells within the subpopulation was between $54.6 \pm 18.9\%$ (CD34^{II}) and $65.0 \pm 17.9\%$ (CD133) (fig. 3c). Interestingly, the functional activity was mainly restricted to cells co-expressing some CD34 epitope and CD133. As shown in figure 3d, over 72% of these subpopulations pumped out Rho123, in contrast to 26.2% (CD34⁺ CD133[−]) and 43.5% (CD34[−] CD133⁺) of corresponding cells missing one stem cell marker ($p \leq 0.001$). Notably, the rare fraction of CD133+ CD34^{I/II/III}− cord blood cells had the lowest ability to exclude the dye.

As was the case with cord blood cells, we found no significant differences with respect to Rho123 exclusion activity between the peripheral blood progenitor populations defined by CD34

Fig. 3. Functional activity of Rho123 efflux of HPC. Typical example: **a** Rho123 staining of CD34^{III}+ cord blood cells after Rho123 uptake. **b** The same sample as in figure 3a after incubation for 40 min at 37 °C. **c** Percentages of Rho123^{dull} cells in cord blood (n = 15) and peripheral blood samples (n = 8) (*p ≤ 0.05, ***p ≤ 0.005 compared to CD133+ cells, ###p ≤ 0.005 compared to peripheral blood). Percentages of Rho123^{dull} cells in cord blood subpopulations (co-)expressing CD34 epitopes and CD133 (*p ≤ 0.05, ***p ≤ 0.005 compared to CD34+ CD133+ cells).

epitope expression. However, peripheral blood cells had lower exclusion activity ($35.5 \pm 11.1\%$ CD34^{III}, $43.6 \pm 11.3\%$ CD34^{II}, fig. 3c) than cord blood cells. Remarkably, the high functional capacity of CD133+ cells from peripheral blood ($68.0 \pm 13.5\%$, $p \leq 0.05$ compared to CD34+ cells) is comparable to that of the corresponding cord blood-derived cells. Their CD34^{III}+ CD133+ subset showed a significantly decreased number of cells with efflux capacity (fig 4a).

Inhibition of Pgp-170 Mediated Rho123 Efflux of Progenitor Cells by Verapamil

Verapamil is a specific inhibitor of Pgp-170. We determined the verapamil-specific inhibition of dye delivery depending on age of donor (umbilical cord blood, children (4–7 years) and adults (>18 years)). Verapamil concentration to block Rho123 uptake was verified by ADR cells. Surprisingly, we could show a clear age-related dependency of protein pump inhibition by verapamil in the CD34+ CD133– population (fig. 4b, 50% in adults, 82% in children, 98% in cord blood). However, in the CD34+ CD133+ populations the Rho123 efflux was completely inhibited, suggesting that the Rho123 efflux activity of this population is exclusively mediated by Pgp170.

Analysis of Pgp-170 Expression on CD34+ and CD133+ Cord Blood Cells

As shown by verapamil inhibition of Rho123 efflux, both CD34+ and CD133+ cord blood cells were effectively blocked. This indicated that the export of the dye is mainly mediated by the *mdr1* gene product Pgp-170. Consequently, we characterised the expression of this verapamil-sensitive transporter with external epitope-specific monoclonal antibodies against Pgp-170 (mAb MRK16, UIC2, 4E3) on CD34/CD133-expressing cell fractions of cord blood HPC. Although we could show that each Pgp-170 mAb binds to cells of the verapamil-sensitive cell line ADR (>90%, human bladder cell carcinoma), we could not detect any staining with the mAb UIC2 on CD34+/CD133+ cord blood cells. Furthermore, only $2.37 \pm 2.76\%$ of CD34^{III}+ and none of CD133+ cells could be marked with the mAb 4E3. However, the expression of the protein pump Pgp-170 could be clearly detected on CD34+ and on CD133+ cord blood cells using the mAb MRK16 ($85.5 \pm 12.0\%$ and $73.1 \pm 12.7\%$, respectively; fig. 5).

It therefore seems likely that the incubation of cells with Rho123 can significantly affect the binding of Pgp-170-specific mAbs.

Fig. 4. a Percentage of Rho123-excluding cells and **b** inhibition of Rho123 efflux by verapamil (25 $\mu\text{mol/l}$) of CD34^{III}/CD133-expressing cells in dependency on age of donor (* $p \leq 0.05$, *** $p \leq 0.01$ compared to cord blood), cord blood $n = 15$, children (4–7 years) $n = 4$, adults (>18 years) $n = 8$.

Discussion

Previous clinical studies have verified that HSC transplants are safe, suitable and therapeutically effective. Bone marrow, mobilised peripheral blood and cord blood were used as source of allogeneic or autologous stem cells. To predict the suitability of a transplant, it is current practice to assess the number of CD34+ cells available related to kilogram body weight of the recipient. However, the reconstitution rate and especially the short- and long-term engraftment are dependent not only on the total CD34+ dose but also on the presence of certain subsets of CD34+ cells [27, 28]. Thus, the expression of homing receptors such as CXCR4 on HSC is a very good marker to predict the homing of re-infused cells, the first step of successful recovery [29]. In order to ensure the long-term outcome of repopulated patients, the application of true HSC with self-renewal and differentiation capacity has to be guaranteed. For this reason, the phenotyping of more primitive HPC closely related to long-term engraftment capacity is essential. This study used multiparametric flow cytometry to analyse subpopulations of CD34+ cells with respect to the co-expression of CD34 epitopes and CD133, their activity to exclude Rho123 and their Pgp-170 expression in umbilical cord and peripheral blood. These markers are strongly correlated with the most immature compartment of HPC.

The classification of mAb to CD34 epitopes is based on differential sensitivity to enzymatic cleavage, tissue reactivity and Western blotting analysis [9]. Some studies using normal HPC and leukaemia blast cells indicated that the most primitive CD34+ stem cells exhibit the most highly sialylated glycoform of CD34 that are detectable with most CD34^I mAb (highly/partially sensitive to neuraminidase, sensitive to glycoprotease). The differentiation of these cells into lineage-committed progenitors is accompanied by the subsequent loss of these sugars [6, 8, 30]. Steen et al. [7] concluded that CD34^{III} epitopes (resistant to neuraminidase and glycoprotease) were

distributed more broadly on CD34+ HPC than CD34^{II} epitopes (resistant to neuraminidase, sensitive to glycoprotease) and that the lack of CD34^{II} epitopes was confined to CD34+ HPC at a late stage of myeloid differentiation. Additionally, it was suggested that CD34^{III}-expressing cells exhibit a higher proliferative or differential potential than CD34^{II}-carrying cells. Under our experimental conditions and concurrent with published data [31–33], it was found that the majority of umbilical cord as well as peripheral blood CD34+ cells express the CD34^{III} epitope. The cord blood CD34^{III} compartment overlapped completely with the CD34^{II} and CD34^I compartments. However, the commonly recommended antibody for selecting CD34+ cells detects only the CD34^{II} epitope, a part of CD34 compartment. Interestingly, a new study investigating the engraftment and the reconstitution of human T cells in immunodeficient mice showed a clear dependency on CD34 epitope selection [34]. CD34^{III}-selected cells (mAb 8G12, commonly used in diagnostic tests) resulted in an increased T cell reconstitution in mice and a higher signal-joint T cell receptor excision circle production in thymocytes, and thymic progenitors showed higher quality with respect to thymic distribution and higher activities of T cell differentiation compared to CD34^{II}-selected cells (QBEnd, prevalently used in clinical graft engineering). To detect the whole compartment of HPC in peripheral blood, our results suggest that a combination of three epitope-specific antibodies should be used. Most of CD34^I and half of CD34^{II}-expressing cells could not be stained with the commonly used CD34^{III}-specific antibody.

A comparative determination of the reactivity to mAb against CD133 and the ability to exclude Rho123 was performed to see whether these markers correlate with specific CD34 epitope patterns or with each other. By using transcriptional profiling analysis [35], (global) gene expression [36–38] and functional analysis [16, 39, 40], many authors have reported that CD133+ cells reflect a more primitive stage of HPC. This was characterised by a high content of scid-re-populating and

Fig. 5. Estimation of relative number of MRK16- and 4E3-stained cells in cord blood HPC subsets. Mononuclear cells of cord blood were stained with Pgp170-specific antibodies (MRK16, 4E3, $n = 17$) in combination with CD34^{III}, CD133 and CD45. **a, b** Typical flow cytometric staining with Pgp170-mAb of CD34⁺ cord blood cells. **c** Percentage of MRK16- and 4E3-marked cells within CD34⁺ and CD133⁺ subpopulations (box plot: median value = horizontal line in the box, 25/75 percentile = box borders; 10/90 percentile = error bars).

long-term culture-initiating cells [15]. Prominin-1-expressing cells were shown to engraft successfully in an animal transplantation model of primary and secondary recipients, suggesting that these cells have long-term re-populating potential [18, 41]. Clinical studies suggested the feasibility of CD133⁺ selection for transplantation [42] and the therapeutic benefit through successful engraftment using haploidentically mismatched peripheral blood cells [43]. Under our experimental conditions, there was no clear correlation between CD34 epitope and CD133 expression on cord blood and peripheral blood cells. Nearly two thirds of each cord blood CD34 epitope-specific subset carry CD133. Previous studies have generally shown CD34⁺ cord blood cells to have a higher level of CD133 co-expression than either peripheral blood (15%) or bone marrow (approximately 20%) [44]. Gene expression profiles of haematopoietic 'stemness' genes of CD133-selected cells of cord blood and peripheral blood emphasised that there are distinct tissue-specific differences [37]. In agreement with other data, a very rare population of CD133⁺ cells lacking extracellular CD34 antigen was found in cord blood. In the study of McGuckin et al. [44], these cells were detected by staining of intracellular stores of CD34. Recent studies, reflecting the broad therapeutic potential of the CD133 compartment, especially the 'younger' (most primitive) CD34⁺ subset, have reported pluripotent characteristics, including induction of long-term haematopoiesis [45], some embryonic stem cell properties [46] and mesenchymal potential [40, 47].

Furthermore, we correlated the expression patterns of progenitor subpopulations with their functional capacity to exclude Rho123. Summarising previous data, self-renewing HPC are distinguished as the Rho123^{dull} subset of HPC, reflecting a balance between accumulation of Rho123 in activated mitochondria [25] vs. efflux by MDR proteins, especially MDR1 and MDR3 [48]. Thus, Uchida et al. [49] described that more than 40% of mice injected with single lineage marker-negative, Rho123 effluxing side population cells showed long-lasting lymphomyeloid reconstitution. Wagner et al. [21] reported that the slow-dividing fraction of cord blood CD34⁺ CD38[−] HPC is associated with primitive function and self-renewal. Genome-wide gene expression analysis of this fraction showed the highest expression levels for CD133, ERG, cyclin G2, MDR1, osteopontin, CLQR1, IFI16, JAK3, FZD6 and HOXA9, a pattern compatible with their primitive function and self-renewal capacity. Consistent with their high gene expression activity of MDR1, the majority of these cells pump out Rho123. In our study, the CD133 compartment of both cord blood and peripheral blood showed the same capacity to exclude Rho123 – in contrast to the CD34 fraction. Interestingly, detailed phenotypic profiling of the cord blood HPC compartment clarified that only cells co-expressing CD133 and CD34 had a very high capacity to exclude the dye Rho123, independent of the CD34 epitope class. Thus, we suggest that the Rho-123^{dull} CD133⁺ CD34⁺ subpopulation of HPC represents the immature HPC associated with long-term

engraftment capacity. Surprisingly, when analysing the efflux capacity of CD133+ CD34– subset, we had to consider that their potential was strikingly restricted compared to the CD133+ CD34+ subset. The efflux capacity of the CD133+ CD34– subset was similar to that of the CD133– CD34+ subset, representing more lineage-committed haematopoietic precursors characterised by lower engraftment [50] and higher frequency of erythroid colony-forming cells [39].

In order to verify the active transporters of Rho123 efflux, we performed inhibition studies using verapamil, pointing to Pgp-170-mediated transport (MDR1 product) [51]. In agreement with published data, the Rho123 uptake of cord blood HPC was mainly determined by Pgp-170 and independent of the expression of CD34 and CD133 antigens [52]. Furthermore, we estimated a clear age-related dependency to inhibit MDR1 function of CD34+ CD133– cells, in contrast to more primitive CD34+ CD133+ cells in which MDR1 function was completely inhibited. Possibly there is an age-related difference in the expression pattern of the Rho123 transporter.

Although using the Rho123 as a fluorescent Pgp-170 substrate yields good and reliable results for detecting Pgp-170 functional activity, the evaluation of the transporter by staining with mAb is recommended. Moreover, the detection of Pgp-170 antigen by immunophenotyping is more rapid, easier to handle and more reproducible. Especially, the Pgp-170-specific mAbs MRK16, UIC2 (depending on conjugation) and 4E3 have been cited as sensitive markers to detect low level of Pgp-170 expression on cell lines and primary cells (leukaemic blasts) [53]. In our study, we realised the detection of Pgp-170 on cord blood CD34+ as well as CD133+ cells by reliable staining with mAb MRK16 while other tested antibodies failed. This is consistent with published data showing that low, but readily detectable level of Pgp-170 expression on normal lymphocytes (NK cells) could be revealed only by MRK16 [53]. This previous study used a 2-hour retention time to assess Rho123 exclusion ability. In the present study, we have achieved a profiling of HPC compartment related to their maturation stage by varying the retention time of Rho123. When comparing the number of Pgp170-expressing HPC with the number of functionally active cells, cord blood CD34+ cells yielded approximately 20% more MRK16+ cells than Rho123 efflux-active cells, in contrast to comparable amounts

of CD133+ cells. This apparent discrepancy probably reflects changes in the efficiency of the Rho123 transporter depending on the stage of maturation of HPC. In agreement with our present data, more immature HPC-like CD133+ cells are characterised by a highly active system, enabling to pump out the dye within a short time period [22]. Thus, under our experimental conditions, the yields of Rho^{dull} cells of CD34+ HPC mainly represented the functionally active, highly efficient CD133+ subset. Committed progenitor cells like those of the CD34+ CD133– compartment are distinguished by lower expression of Pgp-170 and diminished activity. Estimating the Rho123 efflux kinetics, after prolonged time (120 min) $83.9 \pm 12.4\%$ of CD34+ cells pumped out Rho123 (data not shown), which is consistent with the estimated yield of Pgp-170-expressing cells. Therefore, our study showed that MRK16 is very useful for the flow cytometric detection of the functionally active, Pgp-170-expressing HPC compartment.

In summary, multiparametric flow cytometry allows subtyping of the widely used clinical stem cell marker CD34. By combining CD34 with other predictive stem cell markers, we described a new cell subset of CD34+ CD133+ Rho123^{dull} MRK16+ cells, which could be highly associated with self-renewal and differentiation capacity of the stem cell transplant. The enumeration of this HPC fraction could be very helpful to assess the applicability of stem cell transplant.

At present, the counts of nucleated and viable CD34+ cells and of colony-forming units granulocyte-macrophage define the quality of transplant unit. However, these parameters do not permit a precise prediction of long-term engraftment in recipients. To ensure the long-term success of haemotherapy of malignant diseases by stem cell transplantation, it will be necessary to enumerate the more immature HPC subset. To this end, the flow cytometric estimation of an immature CD34+ subpopulation phenotypically characterised by CD133 and Pgp-170 (MRK16+ Rho123^{dull}) expression could contribute to predict the long-term reconstitution of transplanted patients.

Acknowledgments

We thank Michael Cross for helpful discussions and encouragement and Audrey Braun for manuscript preparation.

References

- 1 Eichler H, Burkhart J, Sputtek A, Wiesneth M: Stellungnahme der Sektion Transplantation und Zelltherapie der DGTI (15. August 2005) zur Gewinnung und Langzeitlagerung von autologen und allogenen Stammzellpräparaten aus Nabelschnurblut: Indikationen und Grenzen. *Transfus Med Hemother* 2005;32:274–282.
- 2 Keeney M, Gratama JW, Sutherland DR: Critical role of flow cytometry in evaluating peripheral blood hematopoietic stem cell grafts. *Cytometry A* 2004;58A:72–75.
- 3 Mittag A, Lenz D, Gerstner AOH, Tárnok A: Hyperchromatic cytometry principles for cytomics using slide based cytometry. *Cytometry A* 2006;69A:691–703.
- 4 Tárnok A, Bocsi J, Brockhoff G: Cytomics – importance of multimodal analysis of cell function and proliferation in oncology. *Cell Prolif* 2006;39:495–505.
- 5 Bocsi J, Mittag A, Sack U, Gerstner AOH, Barten MJ, Tárnok A: Novel aspects of systems biology and clinical cytomics. *Cytometry A* 2006;69A:105–108.
- 6 Steen R, Tjonnfjord GE, Gaudernack G, Brinch L, Egeland T: Differences in the distribution of CD34 epitopes on normal haemopoietic progenitor cells and leukaemic blast cells. *Br J Haematol* 1996;94:597–605.
- 7 Steen R, Tjonnfjord GE, Groseth LAG, Egeland T: Characteristics of haemopoietic progenitor cells related to CD34 epitope class expression. *Eur J Haematol* 2000;64:245–251.

- 8 Maynadie M, Gerland L, Aho S, Girodon F, Bernier M, Brunet C, Campos L, Daliphard S, Deney V, Falkenrodt A, Jacob MC, Kuhlein E, LeCalvez G, Moskovtchenko P, Philip P, Carli PM, Faure GC, Bene MC: Clinical value of the quantitative expression of the three epitopes of CD34 in 300 cases of acute myeloid leukemia. *Haematologica* 2002;87:795–803.
- 9 Lanza F, Healy L, Sutherland DR: Structural and functional features of the CD34 antigen: an update. *J Biol Regul Homeost Agents* 2001;15:1–13.
- 10 Gratama JW, Sutherland DR, Keeney M, Papa S: Flow cytometric enumeration and immunophenotyping of hematopoietic stem and progenitor cells. *J Biol Regul Homeost Agents* 2001;15:14–22.
- 11 Shmelkov SV, St Clair R, Lyden D, Rafii S: AC133/CD133/prominin-1. *Int J Biochem Cell Biol* 2005;37:715–719.
- 12 Miraglia S, Godfrey W, Yin AH, Atkins K, Warnke R, Holden JT, Bray RA, Waller EK, Buck DW: A novel five-transmembrane hematopoietic stem cell antigen: Isolation, characterization, and molecular cloning. *Blood* 1997;90:5013–5021.
- 13 Hilbe W, Dirnhofer S, Oberwasserlechner F, Schmid T, Gunsilius E, Hilbe G, Woll E, Kahler CM: CD133 positive endothelial progenitor cells contribute to the tumour vasculature in non-small cell lung cancer. *J Clin Pathol* 2004;57:965–969.
- 14 Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, Henkelman RM, Cusimano MD, Dirks PB: Identification of human brain tumour initiating cells. *Nature* 2004;432:396–401.
- 15 de Wynter EA, Durig J, Cross MA, Heyworth CM, Testa NG: Differential response of CD34+ cells isolated from Cord Blood And Bone Marrow to MIP-1 α and the expression of MIP-1 α Receptors On These Immature Cells. *Stem Cells* 1998;16:349–356.
- 16 Pasino M, Lanza T, Marotta F, Scarso L, De Biasio P, Amato S, Corcione A, Pistoia V, Mori PG: Flow cytometric and functional characterization of AC133+ cells from human umbilical cord blood. *Br J Haematol* 2000;108:793–800.
- 17 Jaatinen T, Hemmoraanta H, Hautaniemi S, Niemi J, Nicorici D, Laine J, Yli-Harja O, Partanen J: Global Gene Expression Profile Of Human Cord Blood-Derived CD133+ cells. *Stem Cells* 2006;24:631–641.
- 18 Yin AH, Miraglia S, Zanjani ED, AlmeidaPorada G, Ogawa M, Leary AG, Olweus J, Kearney J, Buck DW: AC133, a novel marker for human hematopoietic stem and progenitor cells. *Blood* 1997;90:5002–5012.
- 19 Gallacher L, Murdoch B, Wu DM, Karanu FN, Keeney M, Bhatia M: Isolation and characterization of human CD34+Lin– and CD34+Lin– hematopoietic stem cells using cell surface markers AC133 and CD7. *Blood* 2000;95:2813–2820.
- 20 Vodyanik MA, Bork JA, Thomson JA, Slukvin II: Human embryonic stem cell-derived CD34+ cells: efficient production in the coculture with OP9 stromal cells and analysis of lymphohematopoietic potential. *Blood* 2005;105:617–626.
- 21 Wagner W, Ansoorge A, Wirkner U, Eckstein V, Schwager C, Blake J, Miesala K, Selig J, Saffrich R, Ansoorge W, Ho AD: Molecular evidence for stem cell function of the slow-dividing fraction among human hematopoietic progenitor cells by genome-wide analysis. *Blood* 2004;104:675–686.
- 22 Kim M, Moon HB, Spangrude GJ: Major age-related changes of mouse hematopoietic stem/progenitor cells. *Ann N Y Acad Sci* 2003;996:195–208.
- 23 Bunting KD: ABC transporters as phenotypic markers and functional regulators of stem cells. *Stem Cells* 2002;20:11–20.
- 24 Zinovyeva MV, Zijlmans MJM, Fibbe WE, Visser JWM, Belyavsky AV: Analysis of gene expression in subpopulations of murine hematopoietic stem and progenitor cells. *Exp Hematol* 2000;28:318–334.
- 25 Kim MJ, Cooper DD, Hayes SF, Spangrude GJ: Rhodamine-123 staining in hematopoietic stem cells of young mice indicates mitochondrial activation rather than dye efflux. *Blood* 1998;91:4106–4117.
- 26 Sutherland DR, Anderson L, Keeney M, Nayar R, Chin-Yee I: The ISHAGE guidelines for CD34+ cell determination by flow cytometry. *International Society of Hematotherapy and Graft Engineering. J Hematother* 1996;5:213–226.
- 27 Law P, Traylor L, Recktenwald DJ: Cell analysis for hematopoietic stem/progenitor cell transplantation. *Cytometry* 1999;38:47–52.
- 28 Levering WHBM, Sutherland DR, Keeney M, Kraan J, Gratama JW: Haematopoietic stem and progenitor cells: enumeration, phenotypic characterisation, and clinical applications. *Transfus Med Hemother* 2004;31:341–352.
- 29 Kahn J, Byk T, Jansson-Sjostrand L, Petit I, Shvitiel S, Nagler A, Hardan I, Deutsch V, Gazit Z, Gazit D, Karlsson S, Lapidot T: Overexpression of CXCR4 on human CD34+ progenitors increases their proliferation, migration, and NOD/SCID repopulation. *Blood* 2004;103:2942–2949.
- 30 Lanza F, Moretti S, Castagnari B, Latorraca A, Rigolin GM, Bardi A, Castoldi G: Cd34+ leukemic-cells assessed by different CD34 monoclonal-antibodies. *Leuk Lymphoma* 1995;18:25–30.
- 31 Croockewit AJ, Raymakers RAP, Preijers FWMB, Vierwinden G, de Witte TJM: The role of the different CD34 epitopes in detection and positive selection of CD34+ bone marrow and peripheral blood stem cells. *Scand J Immunol* 1998;47:82–90.
- 32 Steen R, Egeland T: CD34 molecule epitope distribution on cells of haematopoietic origin. *Leuk Lymphoma* 1998;30:23–30.
- 33 Sovalat H, Racadot E, Henon P, Fuchs P, Lewandowski H, Billot M: Comparative analysis of class I, II and III epitope-detecting CD34 monoclonal antibodies by quantitative flow cytometry. *Hematol Cell Ther* 1998;40:259–268.
- 34 Otto M, Chen X, Martin WJ, Leung W, Knowles J, Holladay M, Houston J, Handgretinger R, Barfield RC: Selection of stem cells by using antibodies that target different CD34 epitopes yields different patterns of T-cell differentiation. *Stem Cells* 2007;25:537–542.
- 35 Hemmoraanta H, Hautaniemi S, Niemi J, Nicorici D, Laine J, Yli-Harja O, Partanen J, Jaatinen T: Transcriptional profiling reflects shared and unique characters for CD34+ and CD133+ cells. *Stem Cells Dev* 2006;15:839–851.
- 36 Jaatinen T, Hemmoraanta H, Hautaniemi S, Niemi J, Nicorici D, Laine J, Yli-Harja O, Partanen J: Global gene expression profile of human cord blood-derived CD133+ cells. *Stem Cells* 2006;24:631–641.
- 37 Toren A, Bielora B, Jacob-Hirsch J, Fisher T, Kreiser D, Moran O, Zeligson S, Givol D, Yitzhaky A, Itskovitz-Eldor J, Kventzel I, Rosenthal E, Amariglio N, Rechavi G: CD133-positive hematopoietic stem cell ‘stemness’ genes contain many genes mutated or abnormally expressed in leukemia. *Stem Cells* 2005;23:1142–1153.
- 38 He XH, Gonzalez V, Tsang A, Thompson J, Tsang TC, Harris DT: Differential gene expression profiling of CD34+ CD133+ umbilical cord blood hematopoietic stem progenitor cells. *Stem Cells Dev* 2005;14:188–198.
- 39 Freund D, Oswald J, Feldmann S, Ehninger G, Corbeil D, Bornhauser M: Comparative analysis of proliferative potential and clonogenicity of MACS-immunomagnetic isolated CD34+ and CD133+ blood stem cells derived from a single donor. *Cell Prolif* 2006;39:325–332.
- 40 Bonanno G, Perillo A, Rutella S, de Ritis DG, Mariotti A, Marone M, Meoni F, Scambia G, Leone G, Mancuso S, Pierelli L: Clinical isolation and functional characterization of cord blood CD133+ hematopoietic progenitor cells. *Transfusion* 2004;44:1087–1097.
- 41 Handgretinger R, Gordon PR, Leimig T, Chen XH, Buhning HJ, Niethammer D, Kuci S: Biology and plasticity of CD133+ hematopoietic stem cells. *Ann N Y Acad Sci* 2003;996:141–151.
- 42 Lang P, Bader P, Schumm M, Feuchtinger T, Einsele H, Fuhrer M, Weinstock C, Handgretinger R, Kuci S, Martin D, Niethammer D, Greil J: Transplantation of a combination of CD133+ and CD34+ selected progenitor cells from alternative donors. *Br J Haematol* 2004;124:72–79.
- 43 Bitan M, Shapira MY, Resnick IB, Zilberman I, Miron S, Samuel S, Ackerstein A, Elad S, Israel S, Amar A, Fibach E, Or R, Slavin S: Successful transplantation of haploidentically mismatched peripheral blood stem cells using CD133+-purified stem cells. *Exp Hematol* 2005;33:713–718.
- 44 McGuckin CP, Pearce D, Forraz N, Toozee JA, Watt SM, Pettengell R: Multiparametric analysis of immature cell populations in umbilical cord blood and bone marrow. *Eu J Haematol* 2003;71:341–350.
- 45 Kuci S, Wessels JT, Buhning HJ, Schilbach K, Schumm M, Seitz G, Loffler J, Bader P, Schlegel PG, Niethammer D, Handgretinger R: Identification of a novel class of human adherent CD34+ stem cells that give rise to SCID-repopulating cells. *Blood* 2003;101:869–876.
- 46 Baal N, Reisinger K, Jahr H, Bohle RM, Liang O, Munstedt K, Rao CV, Preissner KT, Zygmunt MT: Expression of transcription factor Oct-4 and other embryonic genes in CD133 positive cells from human umbilical cord blood. *Thromb Haemost* 2004;92:767–775.
- 47 Tondreau T, Meuleman N, Delforge A, Dejeneffe M, Leroy R, Massy M, Mortier C, Bron D, Lagneaux L: Mesenchymal stem cells derived from CD133-positive cells in mobilized peripheral blood and cord blood: Proliferation, Oct4 expression, and plasticity. *Stem Cells* 2005;23:1105–1112.
- 48 Zhou S, Morris JJ, Barnes YX, Lan L, Schuetz JD, Sorrentino BP: Bcrp1 gene expression is required for normal numbers of side population stem cells in mice, and confers relative protection to mitoxantrone in hematopoietic cells in vivo. *Proc Natl Acad Sci U S A* 2002;99:12339–12344.
- 49 Uchida N, Dykstra B, Lyons KJ, Leung FY, Eaves CJ: Different in vivo repopulating activities of purified hematopoietic stem cells before and after being stimulated to divide in vitro with the same kinetics. *Exp Hematol* 2003;31:1338–1347.
- 50 Gordon PR, Leimig T, Babarin-Dorner A, Houston J, Holladay M, Mueller I, Geiger T, Handgretinger R: Large-scale isolation of CD133+progenitor cells from G-CSF mobilized peripheral blood stem cells. *Bone Marrow Transplant* 2003;31:17–22.
- 51 Rosati A, Candussio L, Crivellato E, Klugmann FB, Giraldi T, Damiani D, Michelutti A, Decorti G: Bodipy-FL-verapamil: A fluorescent probe for the study of multidrug resistance proteins. *Cell Oncol* 2004;26:3–11.
- 52 Raaijmakers HGP, van den Bosch G, Boezeman J, de Witte T, Raymakers RAP: Single-cell image analysis to assess ABC-transporter-mediated efflux in highly purified hematopoietic progenitors. *Cytometry* 2002;49:135–142.
- 53 Taylor BJ, Olson DP, Ivy SP: Detection of P-glycoprotein in cell lines and leukemic blasts: failure of select monoclonal antibodies to detect clinically significant Pgp levels in primary cells. *Leuk Res* 2001;25:1127–1135.