Rhodamine 123 efflux in human subpopulations of hematopoietic stem cells: Comparison between bone marrow, umbilical cord blood and mobilized peripheral blood CD34+ cells

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Abstract. Hematopoietic stem cells (HSC) can be identified by the expression of the CD34 molecule. CD34+ cells are found in bone marrow (BM), umbilical cord blood (UCB) and in mobilized peripheral blood (PB). CD34+ cells express P-glycoprotein (Pgp), a product of the multidrug resistance (MDR) gene. Pgp activity can be measured by the efflux of the dye Rhodamine 123 (Rho 123) and can be blocked by verapamil. Transport activity in HSC suggests that Pgp could have a functional role in stem cell differentiation. This study compared the number of CD34+ cells with Pgp activity measured by efflux of Rho 123 in the hematopoietic population obtained from different sources. Samples were analysed for their content of CD34+ cells, and BM had a significantly higher amount of CD34+ cells compared to UCB, mobilized PB and normal PB. When the frequency of Rho^{low} cells was studied among the CD34+ population, an enrichment of cells with Pgp activity was observed. The frequency in BM was significantly lower than that in UCB and mobilized PB. The low retention of Rho 123 could be modified by verapamil, indicating that the measurements reflected dye efflux due to Pgp activity. Although UCB and mobilized PB had a lower number of CD34+ cells compared to BM, the total number of CD34+ cells with Pgp activity was similar in the three tissues. The different profiles may indicate the existence of subpopulations of stem cells or different stages of cellular differentiation detected by the extrusion of the dye Rho 123.

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Introduction

Hematopoietic stem cells (HSC) are characterized by their ability to self-renew and differentiate into multipotent progenitors, which develop subsequently into myeloid and lymphoid cells (1,2). The first quantitative *in vivo* study of stem cells was conducted in 1961 by Till and McCulloch (3), demonstrating that a single precursor cell exists in the bone marrow (BM) of adult animals which is capable of both extensive self-renewal and multi-lineage differentiation. HSC can be characterized based on both phenotype and function. The human CD34 protein is a developmental stage-specific surface antigen expressed in hematopoietic stem and progenitor cells, and it has been used for the selection of long-term repopulating cells (4,5). Therefore, CD34+ cells are commonly used in hematopoietic stem cell transplantation. CD34+ cells are found in BM (6), umbilical cord blood (UCB) (7) and in peripheral blood (PB) where they are extremely rare. They can be mobilized to the periphery by means of chemotherapy and/or cytokine treatment, such as G-CSF (granulocyte colony-stimulating factor), increasing their numbers in the circulation (8,9). Therefore, UCB and mobilized PB have become important in the study of hematopoiesis, and they are being used as alternative sources to BM for HSC transplantation (7,10). Despite the lack of a universal protocol for the enumeration of CD34+ cells, there is a general consensus that reliable engraftment can occur in patients receiving at least $2x10^6$ CD34+ cells/kg body weight for mobilized PB (11,12), $0.8\text{-}1.7x10^5$ CD34+ cells/kg for UCB (13,14), and $0.5\text{-}2x10^6$ CD34+ cells/kg for BM (12,15). It is not clear, however, if CD34+ cells from all of these sources represent the same type of cell.

Another functional and phenotypical method of enriching HSC exploits the fact that some cells have evolved a cellular protection mechanism against toxic metabolites and xenobiotics. This mechanism involves the expression of efflux pumps that belong to the ATP-binding cassette (ABC) superfamily of membrane transporters (16). The best studied

transporter, P-glycoprotein (Pgp or ABCB1), is a 170-kDa transmembrane glycoprotein (17) encoded by the multidrug resistance 1 (MDR 1) gene (18,19). Pgp acts as an ATP-dependent efflux pump that prevents the accumulation of many natural substances and anticancer drugs. MDR 1 gene expression is frequently observed in different human tumors and correlates with resistance to chemotherapy (20). In addition to neoplastic cells, a variety of normal human tissues (kidney, colon, blood-brain barrier, liver and hematopoietic tissue) are known to express Pgp at different levels (21), and it is thought to play an important role in removing toxic metabolites from cells (22). In the hematopoietic compartment, Pgp is expressed by peripheral blood lymphocytes, especially $CD56^+$ natural killer and $CD8^+$ cytotoxic T cells, as well as by $CD34^+$ HSC (23,24). Several lines of evidence suggest that Pgp expression is functionally conserved in HSC (23-25). HSC can be identified based on their ability to efflux fluorescent dyes, such as Rhodamine (Rho) 123 (26). Rho 123 is a known substrate for Pgp and has been used extensively as an indicator for Pgp activity (27,28). This transporter molecule can be modulated by a series of substances known as Pgp reversers. Among these the first studied was the calcium-channel inhibitor, verapamil (VP), which blocks Pgp function (29). Transport activity in HSC suggests the possibility that Pgp could have a functional role in stem cell regulation (30). Indeed, other studies have demonstrated that stem cell populations can differ in their ability to accumulate Rho 123 and that this dye can be used to define functionally distinct subpopulations of primitive stem cells (31-33). It was also reported that the hematopoietic population that expresses CD34 and retains low levels of Rho 123 is responsible for the long-term repopulation (31,33,34). The exact phenotype of HSC is still unknown, and additional approaches to further purify HSC can lead to a better characterization of the behavior of these cells from different sources. The aim of the present study was to compare the amount of $CD34^+$ cells with Pgp activity, measured by efflux of Rho 123, in the hematopoietic population obtained from different sources.

Materials and methods

Samples. Human hematopoietic cells from BM (n=14), UCB (n=11), normal PB (n=5) and mobilized PB (n=8, following 10 μg/kg/day of G-CSF-filgrastim treatment for 5 days) of healthy donors were obtained from the Instituto Nacional de Câncer (INCA). This study had the informed consent from the healthy donors, and the protocol was approved by the local ethics committee.

Rhodamine 123 efflux assay and immunophenotyping. The P-glycoprotein activity was determined by means of Rho 123 (Sigma) efflux, as this fluorescent dye is a substrate for Pgp (27,28). Aliquots of cell suspension ($1x10^6$ cells/ml) were incubated with 200 ng/ml of Rho 123 dye in the presence or absence of the Pgp inhibitor, verapamil (VP) (Sigma) at a concentration of 10 μM for 30 min at 37˚C in a humidified atmosphere of air and 5% CO_2. After washing, cells were incubated in a Rho 123-free medium supplemented with 10% foetal bovine serum (Gibco), in the presence or absence of VP for 90 min. Finally, cells were washed and incubated with anti-CD34 phycoerythin (PE)-labeled monoclonal antibody (Becton Dickinson) for 30 min at 4˚C combined with anti-CD45 Peridinin chlorophyll protein (PerCP)-labeled monoclonal antibody (Becton Dickinson) (the pan-leukocyte marker CD45 is differentially expressed in the leukocyte subpopulations). Then, the cells were resuspended in 1 ml of lysing solution (for total lyses of erythrocytes) for 1 min,

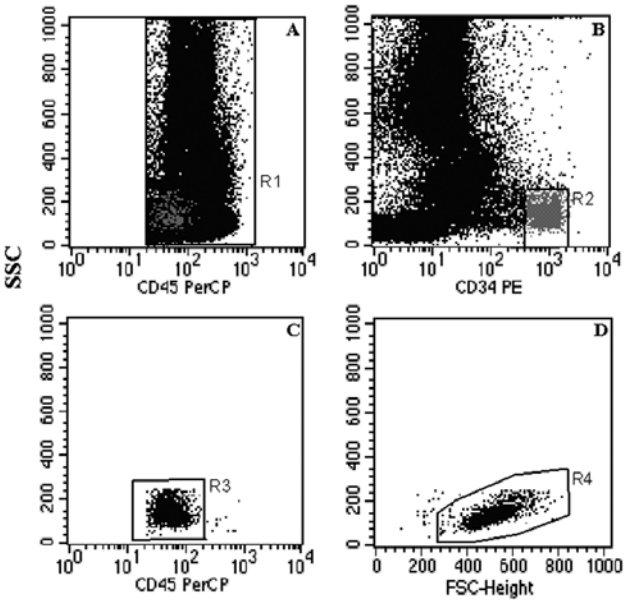

Figure 1. Detection of $CD34^+$ cells. The first stage (A) involved gating $CD45^+$ cells, which was followed (B) by analyzing the $CD34^+$ population among the $CD45^+$ cells. The third stage (C) involved gating the $CD45^{low\ to\ intermediate}$ population among $CD34^+/CD45^+$ cells. The last stage (D) refers to the characteristic light scatter properties of the population gated in C.

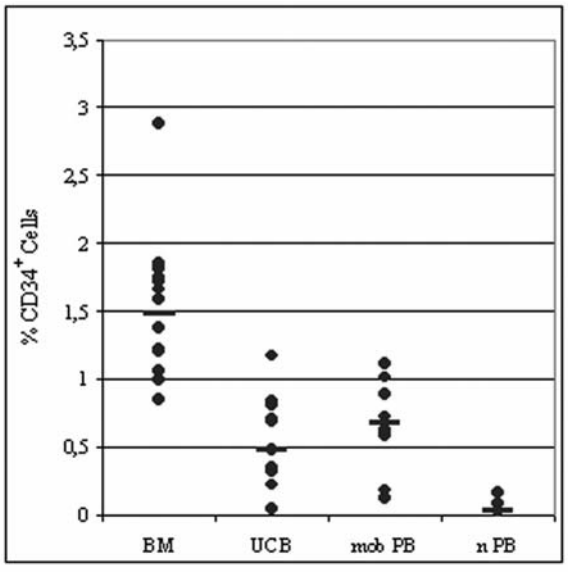

Figure 2. Percentage of $CD34^+$ cells present in different sources of stem cells. BM, bone marrow (n=14); UCB, umbilical cord blood (n=11); mob PB, peripheral blood following treatment with G-CSF (n=8) and n PB, peripheral blood without mobilization (n=5). The dots represent different individuals, and the horizontal line, the median value.

Table I. Comparison of the percentage of CD34+ cells present in the different sources.

	Source			
	BM	UCB	mob PB	n PB
% CD34+ cells/ total leukocytes	1.48	0.48	0.67	0.03
% Rho^{low} cells/ CD34+ cells	26.20	71.11	61.55	56.92
% of cells among leukocytes which are CD34+ and Rho^{low} simultaneously	0.39	0.34	0.41	0.02

Results are expressed as the median of the frequency (%) among the cells of different sources. BM, bone marrow (n=14); UCB, umbilical cord blood (n=11); mob PB, peripheral blood following treatment with G-CSF (n=8) and n PB, peripheral blood without mobilization (n=5).

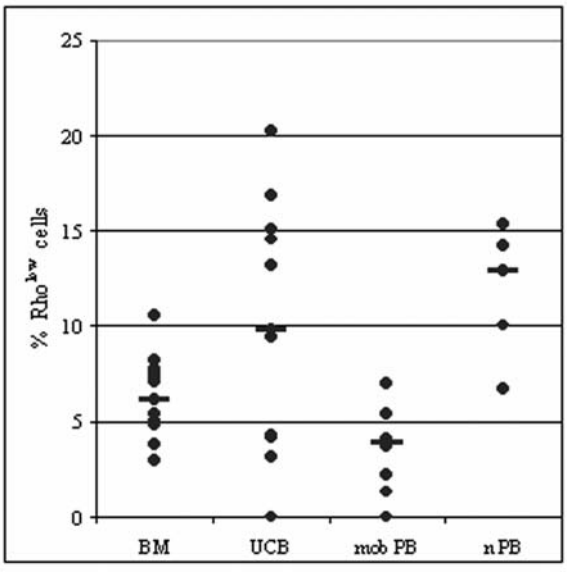

Figure 3. Percentage of cells with Pgp activity (Rho^{low}) present in different sources of stem cells. BM, bone marrow (n=14); UCB, umbilical cord blood (n=11); mob PB, peripheral blood following treatment with G-CSF (n=8) and n PB, peripheral blood without mobilization (n=5). The dots represent different individuals, and the horizontal line, the median value.

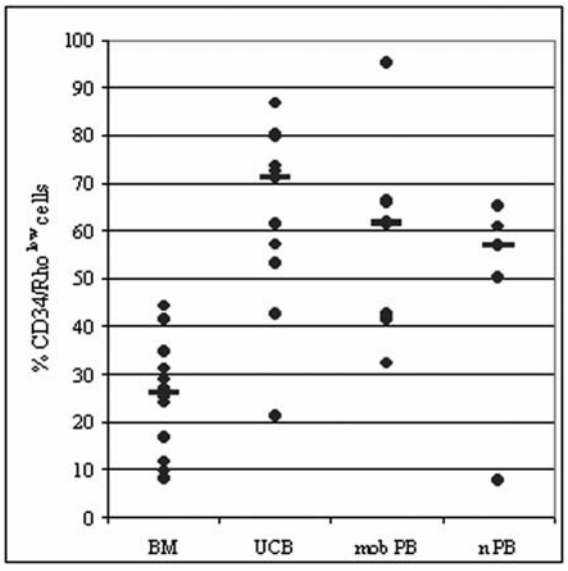

Figure 4. Percentage of CD34+/Rho^{low} cells present in different sources of stem cells. BM, bone marrow (n=14); UCB, umbilical cord blood (n=11); mob PB, peripheral blood following treatment with G-CSF (n=8) and n PB, peripheral blood without mobilization (n=5). The dots represent different individuals, and the horizontal line, the median value.

washed and resuspended in phosphate-buffered saline containing 0.1% sodium azide and 0.5% bovine serum albumin. Cells were used for the analysis of antigen expression and for studies of Pgp activity.

Flow cytometric study. Data acquisition and analysis were performed using a FACScalibur (Becton Dickinson) equipped with a 488-nm argon laser. To investigate dye efflux in CD34+/CD45+ cells, this cell subset was further identified using multiple gating methods, according to the ISHAGE protocol for enumeration of CD34+ HSC (35). CD34+ cells are rare events. A gating strategy that uses light scattering parameters and CD34/CD45 fluorescence aids ensures accurate identification and enumeration. From the gated CD34/CD45 population, the CD34+ cells were identified in a CD34 vs SSC dot plot (Fig. 1). Rho 123 efflux was calculated based on the percentage of dye-effluxing cells in the VP-free experiment, compared with cells treated with VP.

Statistical analysis. The Mann-Whitney non-parametric test was performed to assess the statistical differences between groups, where p-values ≤0.05 were considered statistically significant.

Results

Samples from different sources were analysed for their content of CD34+ cells. The median values of CD34+ cells were 1.48% for BM, 0.48% for UCB, 0.67% for mobilized PB and 0.03% for normal PB (Fig. 2, Table I). BM had significantly higher numbers of CD34+ cells compared to UCB (p=0.0001), mobilized PB (p=0.0002) and normal PB (p=0.0001). There was no difference between UCB and mobilized PB (p=0.27), but normal PB had less CD34+ cells compared to UCB (p=0.0009) and mobilized PB (p=0.0016).

The next step involved establishing the frequency of cells showing Pgp transport activity, i.e., accumulation of low amounts of the dye (Rho^{low}). The median values obtained were 6.14% among BM cells, 9.83% in UCB, 3.91% in mobilized PB and 12.87% in normal PB (Fig. 3). Statistical analysis indicated that there was no difference between BM and UCB (p=0.14), however, BM had significantly more cells compared to mobilized PB (p=0.02) and less cells compared to normal PB (p=0.007). When the proportion of UCB cells was compared, there was a significant difference between this

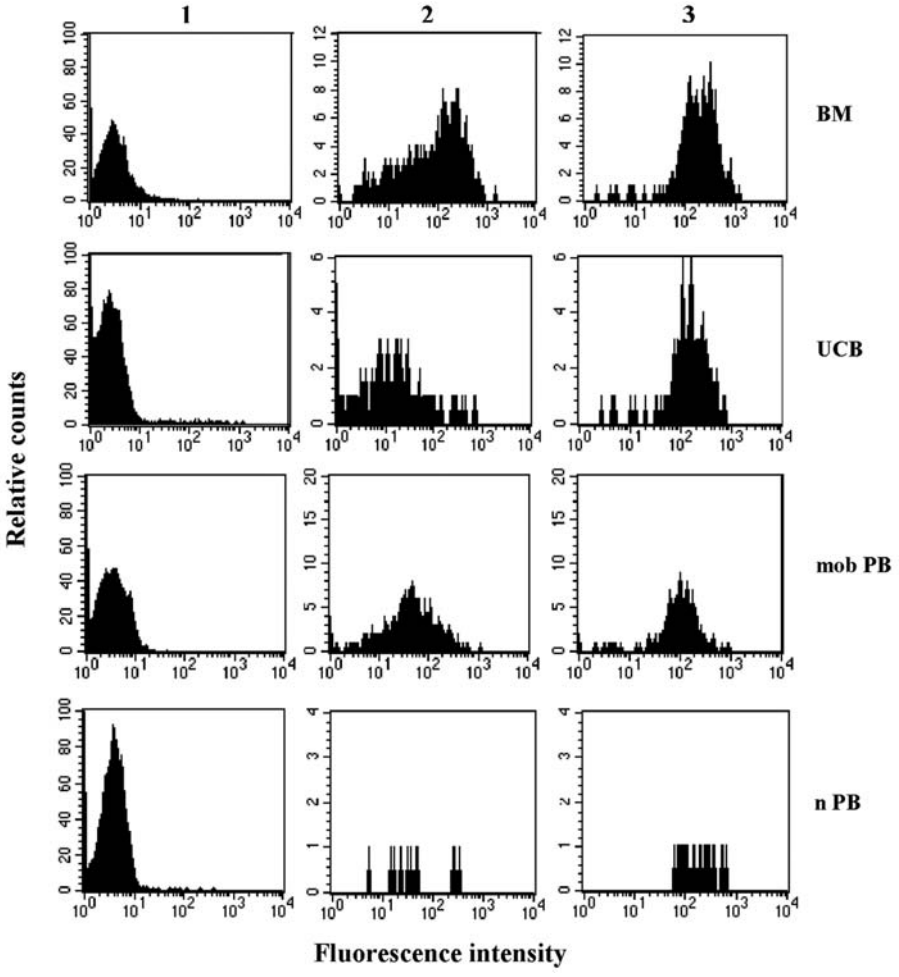

Figure 5. Rho 123 accumulation in CD34+ cells from different sources of stem cells. BM, bone marrow; UCB, umbilical cord blood; mob PB, peripheral blood following treatment with G-CSF and n PB, peripheral blood without mobilization. (1) Auto-fluorescence of cells without Rho 123; (2) cells incubated with 200 ng/ml of Rho 123 and left to extrude the dye and (3) cells incubated with 200 ng/ml of Rho 123 and the Pgp inhibitor verapamil (10 μM). This result is a representative experiment of all the samples tested.

population and mobilized PB (p=0.03) but not between UCB and normal PB (p=0.66). Mobilized PB had a significantly lower number of Rho^{low} cells compared to normal PB (p=0.003).

Mature cells such as CD8 lymphocytes and NK cells have high Pgp activities, therefore it was important to establish the amount of Rho^{low} cells among the immature population. When the frequency of Rho^{low} cells was studied among the CD34+ population (Fig. 4, Table I) it was possible to observe a clear enrichment of cells with Pgp activity; in this case, 26.20% in the BM, 71.11% in UCB, 61.55% in mobilized PB and 56.92% in normal PB. The frequency in the BM was significantly lower than that in the UCB (p=0.0001) and mobilized PB (p=0.0002). When UCB was compared against mobilized PB (p=0.44) and normal PB (p=0.14) no significant difference was observed.

Our results of Rho 123 accumulation and efflux, used to determine Rho^{low} cells, were based on the amount of extrusion that could be blocked by the Pgp inhibitor verapamil (Fig. 5) indicating that differences in the intensity of Rho 123 fluorescence were genuinely due to Pgp activity.

Our data suggest that not all CD34+ cells have Pgp activity. Furthermore, although UCB and mobilized PB had a lower number of CD34+ cells compared to BM, the total amount of CD34+ cells with Pgp activity was similar in the three tissues (Table I). The different profiles may indicate the existence of subpopulations of stem cells or different stages of cellular differentiation detected by the extrusion of the dye Rho 123.

Discussion

The study of hematopoietic stem cells is limited by the lack of specific markers for HSC. Stem cell populations can be highly enriched by a variety of methods that involve cell surface marker expression or functional characteristics. One of these methods involves the use of the fluorescent dye Rho 123 (26), known as a Pgp substrate. In the current study we investigated whether Rho 123 staining in combination with CD34 could be useful in the identification of distinct populations of HSC of the BM, UCB and mobilized PB.

The leukocytes obtained from different sources were analysed with regard to CD34 expression, and the proportion of CD34+ cells obtained from the different sources (BM, UCB, mobilized and normal PB) were in accordance with that described by other authors (8,35-37).

When the frequency of Rho^{low} cells was studied among the $CD34^+$ population of the BM, mobilized and normal PB, our results were consistent with other studies (23,31,38,39). It has been reported (38,39) that $CD34^+$ cells from mobilized PB retain less Rho 123 than those from the BM. A similar trend was observed by us, with a clear difference between the groups. Furthermore, we observed a significant difference between UCB and BM $CD34^+$ cells. However, UCB $CD34^+$ cells displayed an increased capacity of Rho 123 extrusion, much higher than that reported by other groups (32,40). This discrepancy may be a reflection of the time left for Rho 123 extrusion. Zijlmans *et al* (26) described that increasing the time of extrusion leads to an augmentation of the proportion of Rho^{low} cells. On the other hand, Hao *et al* (41) and Mayani *et al* (42) reported an increased proliferative capacity in $CD34^+$ UCB cells compared to BM. Their results and ours strongly indicate that there are significant functional differences between these two populations. Using different assays, various studies have reported the increased engrafting capacity of UCB cells (43-45).

A number of studies have demonstrated that the hematopoietic population that expresses CD34 and retains low levels of Rho 123 is responsible for long-term repopulation (31,33,34). Low levels of Rho 123 retention could not only reflect extrusion of the dye through Pgp activity (27,28), but also a quiescent state of the cells. The dye Rho 123 accumulates in the mitochondria (46) and, therefore, its retention is also a reflection of mitochondria number and activity, explaining why resting cells accumulate less dye. In the present study, the low retention of Rho 123 was able to be modified by the Pgp inhibitor, verapamil, indicating that what was being measured was dye efflux and not mitochondria function.

These results indicate that Rho 123 staining can be used in combination with CD34 antigen to obtain subpopulations of hematopoietic progenitors. Thus, a combination of Rhodamine efflux and phenotypic selection provides an efficient way to enrich immature cells, and these enriched subpopulations should allow us to further characterize the biology and clinical relevance of HSC.

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