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Thy-1 (CDw90) and c-kit receptor (CD117) expression on CD34+ hematopoietic progenitor cells: a five dimensional flow cytometric study

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Abstract

Background and Objective. **CD34+ hematopoietic progenitor cells (HPCs) constitute a heterogeneous population both in size and in immunological features. Lack of CD38, HLA-DR and lineage committed antigens as well as the co-expression of Thy-1 (CDw90) and c-kit receptor (CD117), are able to identify the so-called *stem cells*. A flow cytometric study was carried out to investigate the co-expression of Thy-1 and c-kit receptors, both members of Ig superfamily adhesion molecules, involved in cell to cell and cell to stroma interactions, on bone marrow (BM), mobilized peripheral blood (PB) and human umbilical cord blood (HUCB) CD34+ HPCs.**

Design and Methods. **Lysed whole blood from 15 BM, 25 mobilized PB and 25 HUCB samples were used to perform a five-dimensional flow cytometric evaluation of both CDw90 and CD117 on CD34+ cells.**

Results. **Few CD34+ cells co-expressed Thy-1 antigen in all three compartments (BM: 11.2±7.2%; PB: 6.2±3.6%; HUCB: 6±2.9%; BM vs PB <0.04; BM vs HUCB <0.008; PB vs HUCB ns). c-kit receptor was detected on the majority of CD34+ HPCs, particularly in HUCB (HUCB: 80.7±8.2%; BM: 72.3±13.1%; PB: 64.2±17%; HUCB vs BM <0.03; HUCB vs PB <0.0001; BM vs PB ns). CD34+Thy-1+ and CD34+c-kit+ HPCs generally displayed HLA-DR antigen, as expression of early cell commitment. However, the most immature CD34+Thy-1+HLA-DR– (HUCB: 1± 0.6%; BM: 0.4±03%; PB: 0.7±0.5%; HUCB vs BM <0.0001; BM vs PB <0.04; HUCB vs PB ns) and CD34+c-kit+HLA-DR– HPCs (HUCB: 6.5±4.4%; BM: 6.3±4.8%; PB: 2.2±1.8%; HUCB vs BM ns; BM vs PB <0.0001; HUCB vs PB <0.0001) were mainly detected in HUCB. Finally, the greatest percentage of CD34+Thy-1+c-kit+ cells was found in BM (6.9±4.1%) followed by leukapheretic samples (4.4%±2.7) and then by HUCB (3.7±1.2%; BM vs PB ns; BM vs HUCB <0.001; HUCB vs PB ns).**

Interpretation and Conclusions. **Since the blood release of HPCs is probably due to a perturbation of the adhesive interactions between these cells and the marrow stroma, the different pattern of Thy-1 and c-kit receptor expression on CD34+ HPCs found in the three hemopoietic compartments evaluated can lead to new knowledge about the mobilization kinetics in which the Ig superfamily adhesion molecules are involved.**

Key words: Thy-1, c-kit, CD34, hematopoietic progenitor cells, flow cytometry

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Over the last years there has been a dramatic increase in the number of mobilized peripheral blood (PB) and human umbilical cord blood (HUCB) transplants performed worldwide.[1-5] Previous reports have shown that CD34+ hematopoietic progenitor cells (HPCs) constitute a heterogeneous population both in size and immunological features.[6-14] The absence of CD38, HLA-DR and lineage committed antigens on CD34+ cell surface is able to identify the so-called stem cells. Thy-1 (identified by CDw90) and *c-kit* receptor (identified by CD117) co-expression seems to characterize *true* stem cells, but the exact role of these two molecules in this setting is still not completely elucidated. Both Thy-1 and c-kit receptors belong to the immunoglobulin gene superfamily and along with integrins, selectins, cadherins, and CD44 family glycoproteins, constitute the cell adhesion molecules (CAM). These molecules are involved in cell to cell and cell to stroma interactions, accounting for the restriction to bone marrow (BM) or the mobilization in PB of HPCs.[15,16]

In order to investigate the frequency of Thy-1 and c-kit antigens co-expression both on committed and immature CD34+ HPCs, we performed a five-dimensional flow cytometric study on BM, mobilized PB and HUCB samples.

Materials and Methods

Samples

The flow cytometric analysis was performed on 25 unfractionated heparinized PB leukapheresis samples from patients suffering from neoplastic diseases (13 breast cancer and 12 non-Hodgkin's lymphoma) and undergoing mobilizing high-dose chemotherapy (cyclophosphamide 7 g/sqm) followed by daily subcutaneous administration of G-CSF (5 μg/kg body

weight), on 25 unfractionated heparinized HUCB samples obtained from the umbilical vein immediately after vaginal delivery in uncomplicated term pregnancies, and on 15 unfractionated heparinized BM samples obtained from patients with stage IA Hodgkin or non-Hodgkin's lymphomas.

Cell preparation

One hundred μL of whole PB, BM or HUCB were simultaneously stained with 10 μL fluorescein isothiocyanate (FITC)-, phycoerythrin (PE) or peridinin chlorophyll protein (PerCP)-labeled monoclonal antibodies (MoAbs) recognizing single antigens and CD34, and then incubated for 30 minutes at 4°C in the dark. The following MoAbs combinations were used for flow cytometry: a) anti-HPCA-2 (CD34)-FITC/anti-HLA-DR-PerCP/anti-CDw90-FITC; b) anti-CD34-FITC/anti-HLA-DR-PerCP/anti-CD117-PE; c) anti-CD34-PerCP/anti-CDw90-FITC/anti-CD117-PE; d) anti-CD34-FITC/anti-HLA-DR-PerCP/anti-Leu-17 (CD38)-PE; e) anti-CD34-FITC/anti-HLA-DR-PerCP/anti-LeuM9 (CD33)-PE. All MoAbs were purchased from Becton Dickinson Immunocytometry Systems (BDIS, San José, CA, USA) except for CD117 and CDw90 (Immunotech, Marseille, France).

Mouse IgG1 (γ_1) and IgG2 (γ_2)-FITC, -PE and –PerCP were used as isotypic controls to determine background fluorescence. After red blood cell lysing (Lysing Solution, Ortho Diagnostic) and two washings by centrifugation in phosphate-buffered saline, containing 0.1% sodium azide and 0.5% bovine serum albumin, the samples were analyzed by flow cytometry.

Flow cytometric study

Data were collected on a FACSort flow cytometer (BDIS) equipped with a 15 mW argon laser emitting at 488 nm and Lysis II software. All channels were set to record in the logarithmic mode. An acquisition gate was set according to side light scattering cell properties and fluorescence intensity in order to collect only $CD34^+$ cells, as previously described.[17-20] More than 3000 events were stored in List Mode data files for three-color fluorescence.

Statistics

The Student's t-test was used for statistical comparisons.

Results

Early $CD34^+$ HPCs, displaying CD38- HLA-DR-, or $CD33^-$ HLA-DR- phenotype, were found to be more represented in HUCB and BM than in leukapheretic specimens, as percentage number. Only few $CD34^+$ cells co-expressed Thy-1 antigen in all three compartments, although a higher number resided in BM. On the other hand, c-kit receptor was detected on the majority of $CD34^+$ HPCs, particularly in HUCB. Most of $CD34^+Thy\text{-}1^+$ and $CD34^+c\text{-}kit^+$ cells also co-expressed HLA-DR antigen. Regarding the co-expression of both c-kit receptor and Thy-1 antigens on $CD34^+$ cells, higher percentages of these cells were found in BM and leukapheretic samples than HUCB samples.

Data are depicted in Table 1. Figure 1 (A and B) illustrate contour plots showing a typical co-expression pattern of Thy-1 and c-kit receptor antigens on $CD34^+$ cells in a leukapheretic sample.

Discussion

The CD34 cell surface antigen is expressed on all hematopoietic progenitor cells that constitute a heterogeneous population both in size and in immunological features. Using a three-color fluorescence flow cytometer equipped with a fluorescence-activated cell sorter, Terstappen *et al.* showed that $CD34^+$ cells, separated on the basis of their expression (or lack of expression) of HLA-DR, CD38 and lineage antigens, can be identified and subfractionated in early (the so-called *true* stem cells, $HLA\text{-}DR^-$, $CD38^-$, and lineage $antigen^-$), and committed hematopoietic progenitor cells ($CD34^+HLA\text{-}DR^+CD38^+$) through an intermediate

Table 1. $CD34^+$ cell subsets in tested tissues.

				Statistics (p)		
CD34+ cell subsets	*BM*	*PB*	*HUCB*	*BM vs PB*	*BM vs HUBC*	*HUCB vs PB*
CD34+HLA-DR-CD38-	0.8±0.9 (0.02-9.9)	0.2±0.3 (0.01-1.2)	5.6±5.2 (0.6-20.8)	< 0.02	< 0.02	< 0.0001
CD34+HLA-DR-CD33-	3.3±3.2 (0.2-9.9)	1.8±2.2 (0.05-8.3)	7.3±5.1 (0.8-18.1)	ns	< 0.04	< 0.0001
CD34+Thy-1+	11.2±7.2 (3.5-25)	6.2±3.6 (0.1-11.4)	6.0±2.9 (0.5-12.1)	< 0.04	< 0.008	ns
CD34+HLA-DR-Thy-1+	0.4±0.3 (0.1-0.5)	0.7±0.5 (0.3-0.9)	1±0.6 (0.1-1.4)	< 0.04	< 0.0001	ns
CD34+c-kit+	72.3±13.1 (48.5-89)	64.2±17 (37.4±89)	80.7±8.2 (62.7-91.5)	ns	< 0.03	< 0.0001
CD34+HLA-DR-c-kit+	6.3±4.8 (3.1-9.1)	2.2±1.8 (0.6-3.4)	6.5±4.4 (3.8-7.9)	< 0.0001	ns	< 0.0001
CD34+Thy-1+c-kit+	6.9±4.1 (0.9-12.3)	4.4±2.7 (0.6-6.7)	3.7±1.2 (1-4.3)	ns	< 0.001	ns

Results are expressed as mean percentage number±standard deviation and (range).

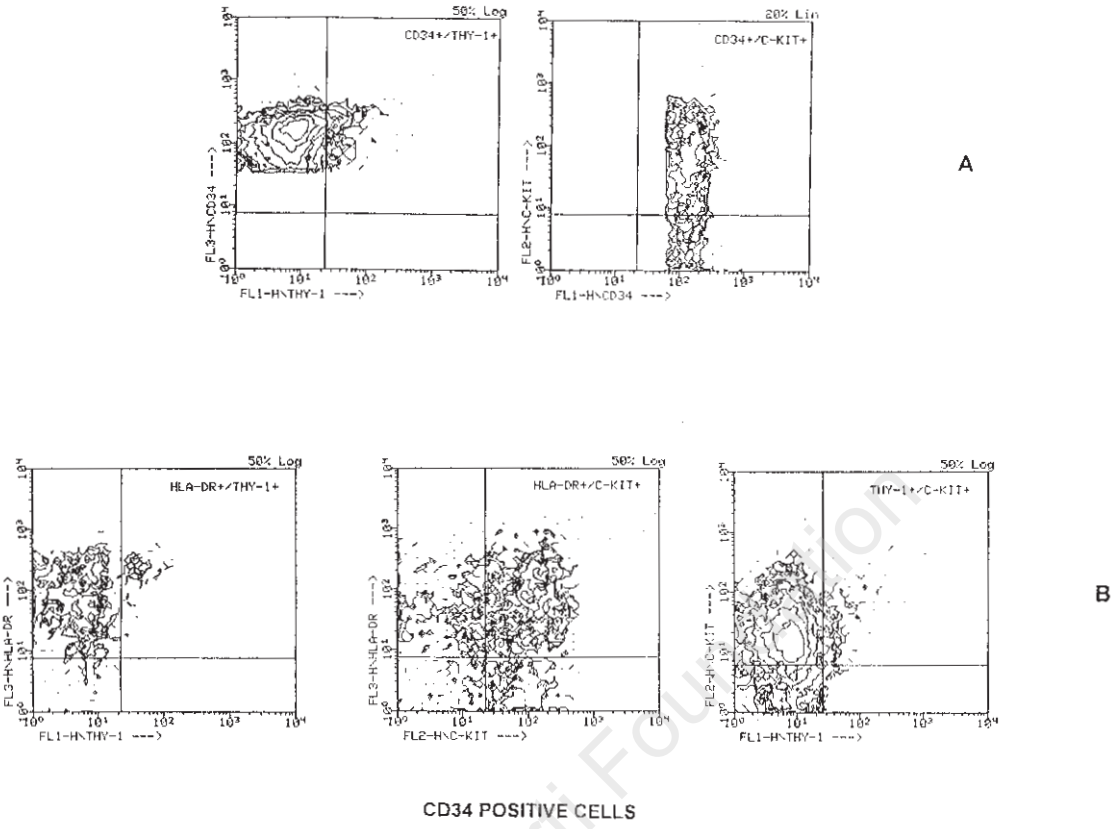

Figure 1. Flow cytometric analysis of leukapheretic CD34+ cells with Thy-1, c-kit receptor and HLA-DR MoAbs (a representative experiment is shown). Cells were labeled with either a PerCP- or FITC-conjugated anti-CD34 and appropriately labeled FITC-Thy-1, PE-c-kit and PerCP-HLA-DR.
On the top line (A) the first two panels from left to right show the typical pattern of co-expression of Thy-1 and c-kit receptor on CD34+ cell surface. On the lower line the first two panels show the pattern of Thy-1/HLA-DR and c-kit/HLA-DR co-expression, while the third panel shows the pattern of Thy-1/c-kit co-expression on CD34+ cell surface.

differentiative step (CD34+HLA-DR+CD38-).[21-23] These data were recently confirmed by other investigators.[24,25] In addition, putative hematopoietic stem cells were shown to co-express Thy-1[26,27] and c-kit receptor[28] antigens, low levels of CD45RO[29] and to be negative for Rhodamine 123 fluorescent staining.[30,31]

HUCB is rich in immature progenitors. Several reports suggested that more primitive cells were present in higher proportion in HUCB than in BM or mobilized PB.[32-34] Despite committed hematopoietic progenitors (colony forming unit-granulocyte macrophage, burst forming unit-erythroid) being found in HUCB in a lower number than in BM and PB, earlier progenitors, evaluated by means of long term culture-initiating cells (LTC-IC), are reported to occur with the same frequency as in BM and in mobilized PB but with increased proliferative ability.[35] In our report, a greater percentage of immunologically defined *immature* progenitor cells (CD34+HLA-DR- CD38- and CD34+HLA-DR-CD33-) was found in HUCB. Furthermore, it is now evident that CD34+Thy-1+ cells are highly enriched for LTC-IC and are transiently present in peripheral blood in the earliest phases of CD34+ cell mobilization.[27,36-39] Nevertheless, we found the highest percentage number of CD34+Thy-1+ cells in BM, while the smallest in HUCB. The latter, however, are enriched in CD34+HLA-DR-Thy-1+ HPCs that probably constitute more immature progenitors than CD34+HLA-DR+Thy-1+ cells. In addition, the hypothesis that CD34+Thy-1+ cells in marrow may functionally differ from the same counterpart in HUCB or mobilized PB in terms of proliferative and differentiative ability must not be discarded.

Thy-1, the smallest member of the immunoglobulin gene superfamily, is a glycophosphatidyl-anchored single-chain 80 kD membrane glycoprotein.[40] Its function is still unknown, but it has been speculated that Thy-1 may mediate a negative signal resulting in the inhibition of primitive cell proliferation.[26] CD34+Thy-1+ cells are capable of initiating and sustaining long-term marrow culture *in vitro*, giving rise to both committed cells, including myeloid, ery-

throid, megakaryocytic, and B-cell precursors, as well as non committed daughter cells. More recently it has been reported that immunoselected CD34$^+$Thy-1$^+$lineage$^-$ stem cells engrafted fetal sheep,[41] and non human primates after gene transfer,[42] opening new perspectives in the field of transplantation.

C-kit receptor and its ligand (named stem cell factor, mast cell growth factor or steel factor) has been reported to play an important role in the development and differentiation of hematopoietic cells.[43] In particular, it appears to promote the survival of the hematopoietic stem cells, whereas later progenitors are influenced by c-kit ligand in synergy with other cytokines.[44-47]

Both Thy-1 and c-kit are members of Ig superfamily molecules mediating, together with others, the complex network of cell to cell and cell to stroma interactions. Recently, several reports supported the hypothesis that the localization of hemopoiesis to BM involves adhesive interactions between primitive hemopoietic cells and the heterogeneous population of stromal cells. In this setting, it is important to note that Thy-1 is also present on marrow stromal cells.[48,49]

Other adhesion molecules are expressed on CD34$^+$ cell surface and are related to different adhesion properties. For example, it has been reported that CD34$^+$LFA-1 (CD11a/CD18 complex) cells contain committed progenitors, whereas CD34$^+$CD33$^-$LFA-1$^-$ cells are enriched in LTC-IC.[50]

The hypothesis that the overshoot of CD34$^+$ HPCs in PB, induced by cytokines and/or chemotherapy, is associated with the upregulation of the ligand for c-kit, the stem cell factor (SCF), within the hemopoietic microenvironment, is supported by the observation that the SCF is a powerful downregulator of c-kit on BM CD34$^+$ cells *in vitro*[51,52] thus suggesting a key role for SCF in mobilization kinetics. The change in expression of this molecule occurs in the BM before the egress of CD34$^+$ HPCs in PB.[15] According to these data, in the present work c-kit levels were found to be lower on mobilized PB CD34$^+$ cells than either in BM and HUCB.

Furthermore, in our hands both CD34$^+$Thy-1$^+$ and CD34$^+$c-kit$^+$ HPCs co-expressed HLA-DR and, differently from data described in another report,[53] CD34$^+$Thy-1$^+$ cells were also found to be predominantly c-kit$^+$ and were confined to HLA-DR bright subpopulations (Figure 1). The number of phenotypically defined CD34$^+$Thy-1$^+$c-kit$^+$ cells was percentually higher than CD34$^+$CD38$^-$HLA-DR$^-$ cells in BM and leukaphereses but not in HUCB samples. This latter population displayed the stem cell phenotype thus suggesting that CD34$^+$Thy-1$^+$c-kit$^+$ does not represent a homogeneous population of immature progenitors, as was previously considered.[53] However, it has been recently reported that with multicolor flow cytometry and cell sorting, CD34$^+$c-kit$^+$ stem cell antigen (Sca)-1$^+$lineage$^-$ murine hematopoietic cells were able to determine long-term multilineage reconstitution in lethally irradiated mice.[54] These data suggest that the hypothesis of the existence of CD34- HPCs must not be discarded.

In conclusion, a better understanding of the complex mechanisms responsible for the trafficking of CD34$^+$ HPCs in the hemopoietic compartments will be possible by means of new knowledge about the antigenic and related functional profile of CD34$^+$ HPCs. Cell adhesion molecules are involved in these mechanisms as signaling molecules. In this setting, a key role could be played by Thy-1, c-kit receptor and its ligand. Further investigations are needed to clarify their role in order to optimize mobilization, yield, as well as the post collection strategies such as positive or negative selection of CD34$^+$ HPCs and gene therapy. In particular, CD34$^+$Thy-1$^+$lineage$^-$ HPCs, which are highly enriched in LTC-IC, can be used as grafts for cancer patients and hematopoietic cell-based gene therapy.[55] In fact, CD34$^+$Thy-1$^+$lineage$^-$ cell subpopulation selection has been shown to be an effective means of removing tumor cells from autografts.[56,57]

Contributions and Acknowledgments

GDA formulated the design of the study and carried out the flow cytometric assays. He was also responsible for the interpretation and writing of the paper. PM was responsible for statistical analysis and wrote the paper with GDA. NC performed statistical analysis with PM and carried out the flow cytometric assays with GDA. MC was responsible for the conception of the study, interpretation, and the writing of the paper with the other authors.

Disclosures

Conflict of interest: none.

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