original paper
Haematologica 1996; 81:216-223

FLOW CYTOMETRIC CHARACTERIZATION OF CD34+ HEMATOPOIETIC PROGENITOR CELLS IN MOBILIZED PERIPHERAL BLOOD AND BONE MARROW OF CANCER PATIENTS

Giovanni D'Arena, Nicola Cascavilla, Pellegrino Musto, Mario Greco, Lazzaro Di Mauro, Angelo Michele Carella, Nicola Dello Iacono,* Mario Carotenuto*

*Division of Hematology and *Transfusional Service, IRCCS "Casa Sollievo della Sofferenza" Hospital, 71013 San Giovanni Rotondo, Italy*

ABSTRACT

Background. **Hematopoietic progenitor cells (HPC), identified by expression of the CD34 surface antigen, show morphological and phenotypic heterogeneity in bone marrow (BM) and peripheral blood (PB).**

Methods. **CD34+ HPC subpopulations present in 18 PB leukaphereses after high-dose chemotherapy in cancer patients and in 11 BM samples from patients with stage IA lymphoma were characterized. In order to identify CD34+ HPC subsets within these two compartments, the expression of lineage- or activation-associated antigens and the c-kit gene product (CD117) was studied by flow cytometry, using a large panel of monoclonal antibodies (MoAb) in double labelling.**

Results. **We observed a higher proportion of CD34+/CD13+ and CD34+/CD33+ cells (myeloid commitment) in harvested leukapheresis products than in BM. On the contrary, a higher percentage of CD34+/CD10+ and CD34+/CD19+ cells (B-lymphoid commitment) was found in BM. The percentage of the most immature subset of CD34+ HPC (CD38- and HLA-DR-) was also higher in BM than in mobilized PB. No differences in proportions were found with respect to the expression of CD14, CD15, CD45RA (myeloid commitment), CD2, CD5, CD7 (T-lymphoid commitment), CD117, CD71 and CD45RO antigens. In terms of absolute values, however, significantly higher amounts of CD34+ HPC co-expressing CD13, CD33, CD5, CD7, CD71, CD117, CD45RA, CD45RO were detected in leukaphereses than in BM. The absolute number of immature HPC (CD34+/CD38- and CD34+/HLA-Dr-) was also significantly increased in mobilized PB.**

Conclusions. **Our data confirm the heterogeneous phenotypic profile of HPC, thus supporting the hypothesis that different CD34+ subpopulations may have clinical relevance on the rapidity and long-term durability of engraftment in patients who undergo high-dose chemotherapy followed by rescue with HPC. We also demonstrated that mobilized PB is a particularly rich source of both primitive and committed HPC, more than BM.**

Key words: hematopoietic progenitor cells, CD34, peripheral blood stem cell transplantation, flow cytometry, leukapheresis, bone marrow

CD34, a heavily glycosylated membrane molecule, is characteristically expressed on early and committed hematopoietic progenitor cells (HPC).[1,2] This antigen is present on 1-3% of normal bone marrow (BM) cells, on 0.01-0.1% of peripheral blood (PB) cells and on 0.1-0.4% of cord blood (CB) cells. Marked increases of PB CD34+ cells occur during the recovery phase after myelosuppressive chemotherapy and/or growth factor administration.[3,4] These cells, which can be estimated by flow cytometry, collected on a large scale by leukapheresis and cryopreserved for transplantation, are able to engraft and to reconstitute hemopoiesis after myeloablative therapy.[5,6] PB HPC transplantation is increasingly used for treating

Correspondence: Giovanni D'Arena, MD, Division of Hematology, IRCCS "Casa Sollievo della Sofferenza" Hospital, 71013 San Giovanni Rotondo, Italy. Tel. international +39.882.410539. Fax. international +39.882.411705.
Received January 15, 1996; accepted March 27, 1996.

high-risk malignancies such as leukemia, lymphoma, myeloma or solid tumors.[7] In fact, PB HPC transplant usually provides earlier platelet and neutrophil recovery, less need for blood products and parenteral antibiotics and shorter hospitalization than BM rescue.[8,9] The enormous number of committed HPC is likely to account for the extremely rapid hematopoietic recovery following PB cell transplantation. On the other hand, the large absolute number of primitive HPC, although decreased percentagewise with respect to BM, is likely to account for the long-term durability of the hematopoietic system reconstituted by PB cell transplantation.[10] This latter notion has literally abolished the use of BM in favor of PB for HPC transplantation.

Since the CD34+ cell population is extremely heterogeneous both in size and surface expression of activation- and lineage-specific antigens,[11-13] in the present investigation a flow cytometric assay of CD34+ cells was performed on both mobilized PB and BM from patients with neoplastic disorders, in order to evaluate the amount and phenotypic profile of the CD34+ subpopulations.

Materials and Methods

Samples

Eighteen unfractionated heparinized PB leukapheresis samples from patients suffering from neoplastic diseases (6 breast and 1 testicular cancer, 6 non-Hodgkin's lymphoma, 5 multiple myeloma) and undergoing mobilizing high-dose chemotherapy (cyclophosphamide 4 or 7 g/sqm) followed by daily subcutaneous administration of G-CSF (5 μg/kg body weight), and eleven unfractionated heparinized BM samples obtained from patients with stage IA Hodgkin or non-Hodgkin's lymphomas were analyzed. The mean absolute number of mononuclear cells was 38.17×10^9/L (range 18.53-117.64×10^9/L) in the leukapheresis products, and 16.85×10^9/L (range 14.68-23.1×10^9/L) in BM. The normal BM of a donor and that of a patient with acute myeloid leukemia in complete remission, used, respectively, for syngeneic and autologous BM transplants, were also selected and analyzed for single comparisons just before *in vivo* infusion. The MoAb used in double labelling with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-labeled MoAb 8G12 directed against CD34 (HPCA-2) are listed in Table 1.

Preparation of cells

One hundred μL of whole mobilized PB or BM were simultaneously incubated at 4°C in the dark for 25 minutes with 10 μL FITC- or PE-labeled MoAb recognizing single antigens and CD34. 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 (Becton Dickinson) 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. In order to obtain a high

Table 1. Monoclonal antibodies used in the study.

MoAb	*Cellular specificity*	*Source*
CD34 (HPCA-2)	Hemopoietic progenitors	BD
HLA-DR (OK-DR)	Activated progenitors	OD
CD38 (Leu-17)	Activated progenitors	BD
CD71 (transferrin receptor)	Activated progenitors	BD
CD117 (c-Kit receptor)	Stem cell factor receptor	IT
CD45RA (Leu-18)	Myeloid precursors	BD
CD45RO (Leu-45RO)	Early progenitors, erythroid precursors	IT
CD2 (Leu-5b)	T-lymphoid precursors	BD
CD5 (Leu-1)	T-lymphoid precursors	BD
CD7 (Leu-9)	T-lymphoid precursors	BD
CD10 (OK-BCalla)	B-lymphoid precursors	OD
CD19 (Leu-12)	B-lymphoid precursors	BD
CD13 (Leu-M7)	Myeloid precursors	BD
CD33 (Leu-M9)	Myeloid precursors	BD
CD14 (Leu-M3)	Myeloid precursors	BD
CD15 (Leu-M1)	Myeloid precursors	BD
CD41 (GpIIb/IIIa)	Platelet precursors	IT

BD: Becton Dickinson; OD: Ortho Diagnostic; IT: Immunotech;

number of CD34+ events for phenotyping CD34+ cells, an acquisition gate was set according to side light scatter and fluorescence intensity so as to collect only cells with CD34 fluorescence signals, as shown in Figure 1. More than 3000 positively gated events were stored in list mode data files and analyzed for two-color fluorescence.

Statistics

The Student's t-test was used, as appropriate, for statistical analysis.

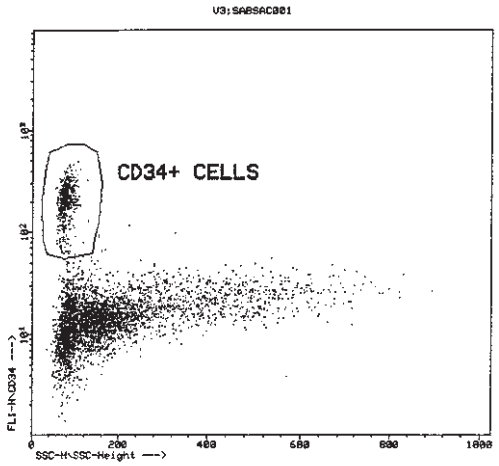

Figure 1. Dot-plot displaying CD34+ expression versus side scatter (granularity). To obtain a maximum number of CD34+ events, an acquisition gate was set according to fluorescence intensity and side light scatter.

Results

The mean total percentage of CD34+ cells in mobilized PB and BM was 4.6%±2.5 and 0.9±0.3, respectively ($p < 0.0001$). The mean absolute number/μL of CD34+ cells was 1965±1981 in PB and 161.9±53.4 in BM ($p < 0.003$). Figure 2 illustrates some examples of CD34 co-expression with other surface antigens tested on HPC from leukaphereses and BM.

Myeloid commitment

Mobilized PB showed a higher proportion and a higher absolute number of both CD34+/CD13+ and CD34+/CD33+ cells than BM. The difference was statistically significant (Figure 3A and 3B). We found no relevant difference between the two compartments with respect to the co-expression of CD14, CD15 and CD41, either in proportional or absolute terms (Figure 3A and 3B).

Lymphoid commitment

A comparison between the percentage of CD34+ cells co-expressing B- and T-lymphocyte-associated cell surface molecules in PB and BM is shown in Figure 4A. A significantly higher percentage of CD34+/CD10+ and CD34+/CD19+ cells (indicating B-lymphoid commitment) was found in BM. However, the absolute number of these B-precursors was similar in PB and BM (Figure 4B). No statistical differences were detected in a proportional analysis of the small subset of CD34+ HPC co-expressing the T-antigens CD2, CD5 and CD7; however, the absolute number of CD34+/CD5+ and CD34+/CD7+ cells was slightly, but significantly, increased in mobilized PB (Figure 4A and 4B).

Other antigens

The majority of CD34+ cells expressed the transferrin receptor (CD71), both in mobilized PB and BM. Moreover, no statistical difference was found between the two compartments with respect to the percentage of CD45RA, CD45RO and CD117 expression on CD34+ cells. The percentage of CD34+/HLA-DR− and CD34+/CD38− primitive progenitor cells was significantly increased in BM (Figure 5A). However, the absolute number of all these subsets of CD34+ cells was significantly higher in mobilized PB than in BM (Figure 5B).

Light scatter properties

With regard to their light scatter properties, CD34+ cells of both sources were heterogeneous and confined within the lymphoid blast region (Figure 6). We arbitrarily divided this population into two regions: a smaller one (R1) containing cells with low forward (FSC) and very low side scatter (SSC), and a second region (R2) containing intermediate to high forward and low side light scattering cells. Regarding the FSC/SSC distribution of the different antigens tested, the most immature CD34+ HPC (HLA-DR−, CD38− and lineage-antigen negative) were confined to region R1.

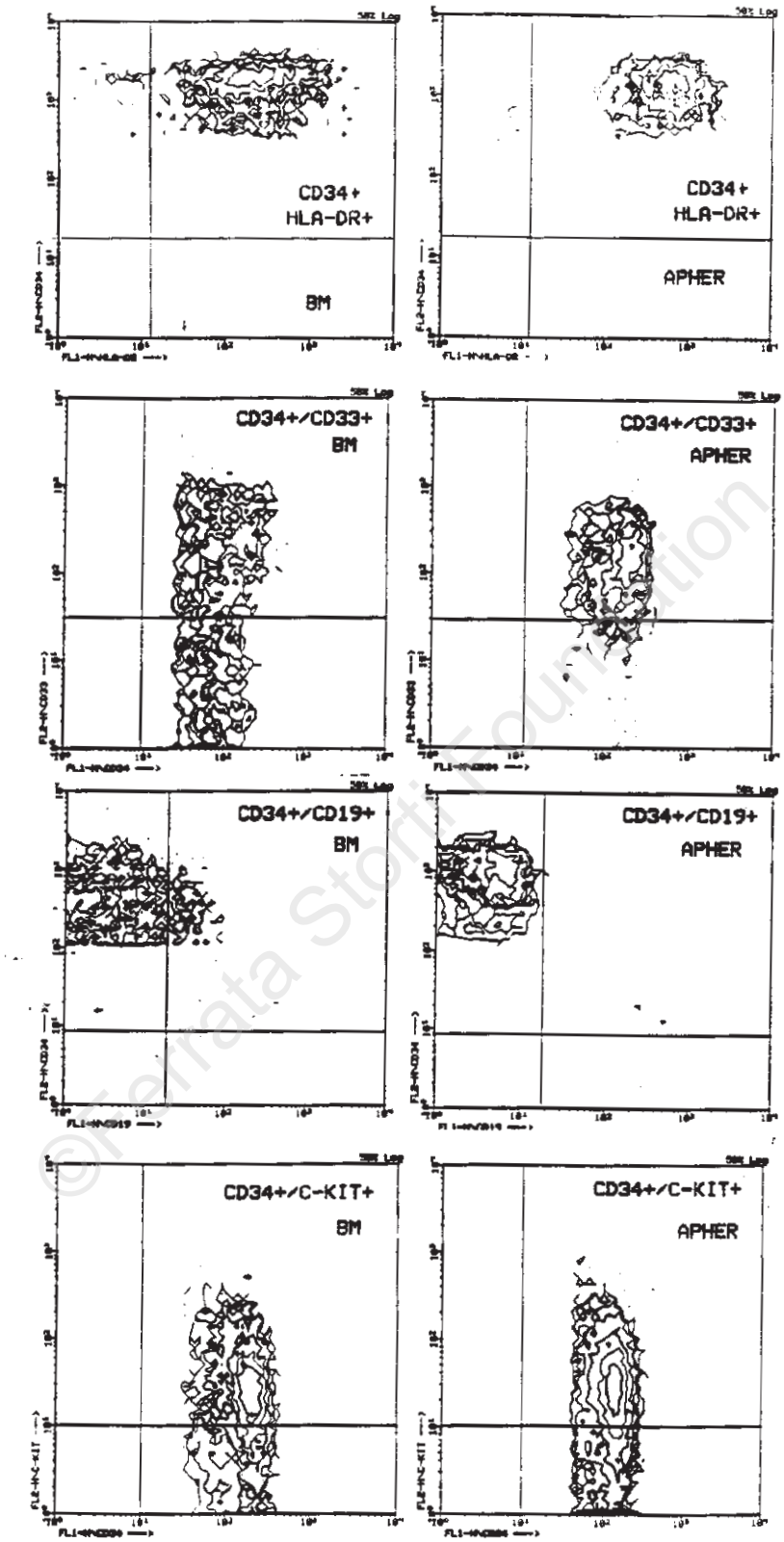

Figure 2. Contour plots showing some examples of CD34 (PE or FITC) co-expression with HLA-DR (PE), CD33 (PE), CD19 (FITC) and c-kit (PE) in BM (left) and mobilized PB (right).

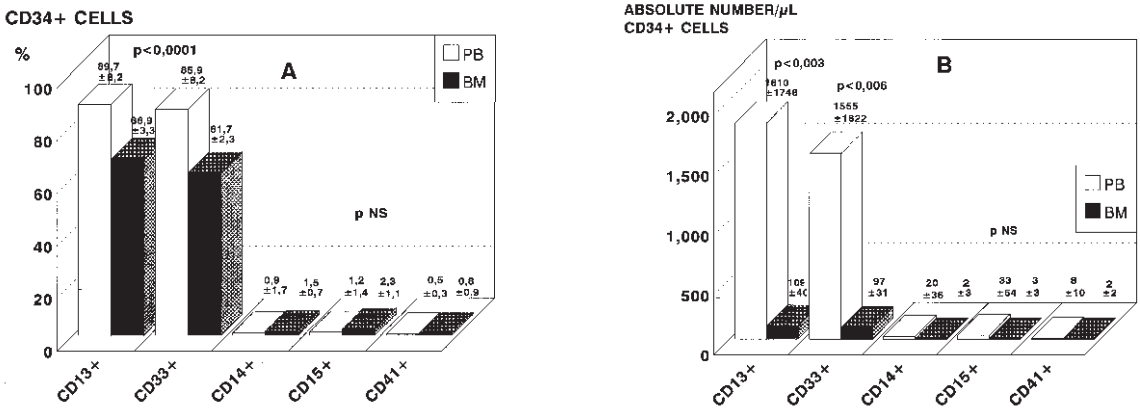

Figure 3. Bar graphs showing mean proportional (A) and absolute (B) values of myeloid antigen co-expression on CD34$^+$ cells in mobilized PB and BM.

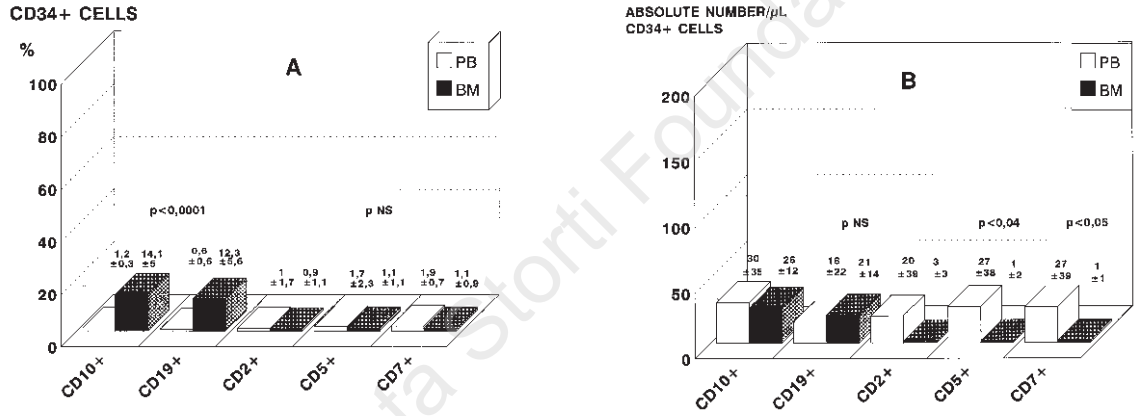

Figure 4. Bar graphs showing mean proportional (A) and absolute (B) values of lymphoid antigen co-expression on CD34$^+$ cells in mobilized PB and BM.

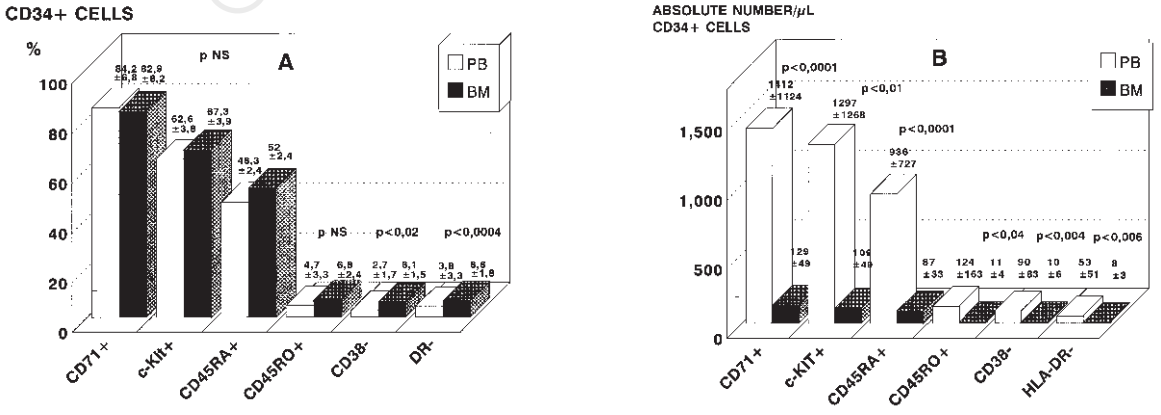

Figure 5. Bar graphs showing mean proportional (A) and absolute (B) values of other antigen co-expression on CD34+ cells in mobilized PB and BM.

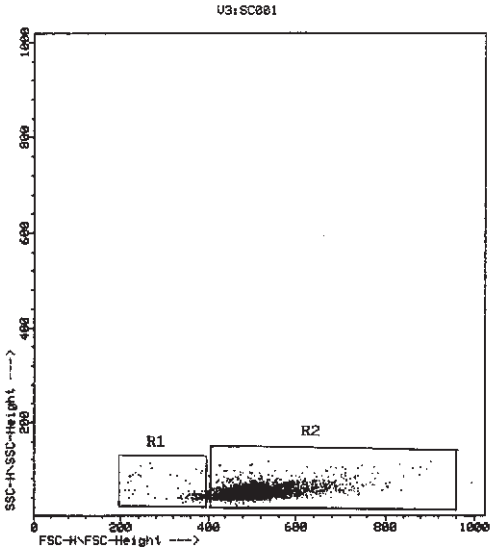

Figure 6. Representative dot plot of a typical profile (forward versus side light scattering) of CD34+ cells. According to light scatter properties, the CD34+ cells were divided into two regions (R1 and R2).

Discussion

Our data confirm that the CD34 molecule is expressed on HPC in mobilized PB and BM from cancer patients, but some significant differences were revealed by the heterogeneous co-expression of other antigens. In particular, both primitive and committed HPC were found in PB, as suggested by previous culture and immunological studies.[14-19] Moreover, we found a direct relationship between light scattering properties and degree of differentiation of CD34 cells.

Leukaphereses were characterized by a higher number of CD34+ cells with co-expression of myeloid antigens (CD13+ and CD33+) than BM, both proportionally and in absolute terms. This is in agreement with other reports,[14-16,19] and supports the hypothesis that the rapid hematologic recovery after PB HPC transplantation is probably due to the presence of a very large number of CD34+ cells with advanced myeloid commitment. No differences in the expression of other *mature* myeloid antigens (CD14, CD15 and CD41) were found.

Although CD10 and CD19 (B-lineage commitment) expression was more frequent on BM CD34+ cells, no statistical difference was seen in absolute terms with respect to mobilized PB. By contrast, a moderate increase in the absolute number of CD34+ cells co-expressing the T-cell antigens CD5 and CD7 was observed in leukaphereses. Whether this difference may be relevant in the immunological recovery of patients transplanted with mobilized PB or BM remains to be established.

Recently, Terstappen and Huang demonstrated the existence in fetal BM of a very small subset of CD34+ HPC that generate both hematopoietic and stromal cell lineages (the *true* common stem cell, as defined by the absence of CD38 and HLA-DR on their surface).[20-22] Further studies have supported this original observation.[23,24] This cell subset seems to be especially numerous in CB.[25-28] In our study we found a small number of these cells proportionally more represented in BM; however, the absolute values of these *primitive* HPC were significantly higher in mobilized PB, as already reported.[29,30] This suggests that the long-term stability of hematopoiesis after peripheral HPC transplant is probably assured by the significant presence of these cells in mobilized PB.

CD71 identifies the transferrin receptor, which is highly expressed on early erythroid progenitors and (at lower levels) by dividing cells, since iron is a basic requirement for proliferation.[31] In our study, the majority of both PB and BM CD34+ cells were found to co-express CD71, thus indicating that they were potentially clonogenic cells. The common leukocyte antigen (CD45) is a highly glycosylated cell surface protein expressed exclusively on cells of the hematopoietic system.[32] CD45RO and CD45RA (two isoforms of CD45) expression on CD34+ cells is mutually exclusive. Thus CD34+ cells can be divided into two distinct subpopulations on the basis of CD45 isoform expression. The majority of primitive CD34+ cells co-express CD45RO; when they differentiate along the erythroid lineage, the total level of CD45 expression decreases. CD45 disappears from mature erythroid cells. Myeloid commitment coincides with the loss of CD45RO and expression of CD45RA.[33] In our experience, only a small number of CD34+ cells were also found to be CD45RO+. A higher percentage co-expressed the

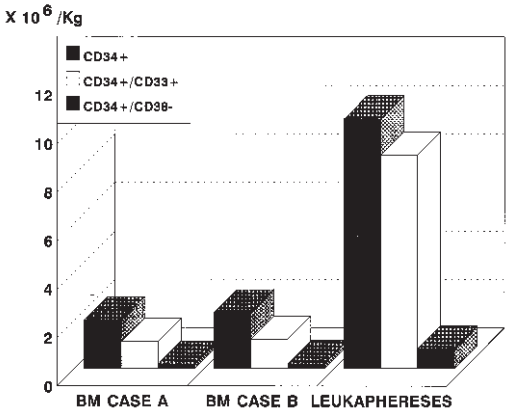

Figure 7. Absolute values of total ($CD34^+$) myeloid-committed ($CD34^+/CD33^+$) and primitive ($CD34^+/CD38^-$) HPC present in the BM of two representative subjects as compared to mobilized PB. Case A was an acute myeloid leukemia patient in complete remission after chemotherapy whose BM was used for autologous transplantation. Case B was a healthy donor for a syngeneic transplant. Both subjects had received G-CSF before BM aspiration. The third series of columns refers to the mean values of the total number of PB $CD34^+$ HPC collected in 14 patients (mean number of leukaphereses per patient: 2.4). The absolute values reported represent the real number of $CD34^+$ HPCs infused.

CD45RA antigen in PB as well as in BM without significant differences, but the absolute value of both these subpopulations was significantly higher in PB. CD117 recognizes the cell surface molecule encoded by the proto-oncogene c-kit, the receptor for stem cell factor, an important molecule that regulates early events occurring during human hematopoiesis.[34,35] This antigen interacts synergistically with a number of cytokines to augment directly the proliferative capacity of primitive human hematopoietic progenitor cells.[36,37] In our study, CD117 divided the $CD34^+$ progenitor cell population into two subsets: a larger one co-expressing c-kit, and a smaller one showing no reactivity to the c-kit antibody. There were no differences in the percentages of these cells in BM and PB leukaphereses; however, $CD34^+/CD117^+$ cells were once again significantly more represented in absolute values in PB.

In conclusion, the most striking differences observed regarding the phenotypic profiles of $CD34^+$ cells were: 1) a high percentage of *myeloid* $CD34^+$ cells co-expressing CD33 and CD13 molecules in PB, which resulted in a very high absolute number of these cells in leukaphereses; 2) a moderate increase in the absolute number of *T-committed* precursors in PB with respect to BM; 3) a higher proportion of less differentiated ($CD38^-$ and HLA-DR^-) $CD34^+$ cells in BM than in PB; however, the absolute number of these primitive HPC was significantly higher in PB. These quantitative differences, in particular those regarding the content of early and committed $CD34^+$ cells, were even more evident when selected final pre-transplant products were analyzed (Figure 7). On this basis, the immunological characteristics of $CD34^+$ cells present in PB and BM could significantly influence the engraftment phase and hematopoietic recovery in cancer patients treated with high-dose chemotherapy followed by HPC rescue.

References

1. Greaves MF, Brown J, Molgaard HV et al. Molecular features of CD34: a hemopoietic progenitor cell-associated molecule. Leukemia 1992; 6:31-6.
2. Sutherland DR, Keating A. The CD34 antigen: structure, biology and potential clinical applications. J Hematother 1992; 1:115-29.
3. Siena S, Bregni M, Brando B, Ravagnani F, Bonadonna G, Gianni M. Circulating of CD34+ hematopoietic stem cells in the peripheral blood of high-dose cyclophosphamide-treated patients: enhancement by intravenous recombinant human granulocyte-macrophage colony-stimulating factor. Blood 1989; 74:1905-14.
4. Gianni AM, Siena S, Bregni M, et al. Granulocyte-macrophage colony-stimulating factor to harvest circulating haemopoietic stem cells for autotransplantation. Lancet 1989; 2:580-5.
5. Siena S, Bregni M, Brando M, et al. Flow cytometry for clinical estimation of circulating hematopoietic progenitors for autologous transplantation in cancer patients. Blood 1991; 77:400-9.
6. D'Arena G, Scalzulli P, Musto P. Circulating hematopoietic progenitor cells in leukapheresis: flow cytometric estimation. Haematologica 1996; 81:95-6.
7. Craig JIO, Turner ML, Parker AC. Peripheral blood stem cell transplantation. Blood Rev 1992; 6:59-67.
8. Elias AD, Ayash L, Anderson KC, et al. Mobilization of peripheral blood progenitor cells by chemotherapy and granulocyte-macrophage colony-stimulating factor for hematologic support after high-dose intensification for breast cancer. Blood 1992; 79:3036-44.
9. To LB, Roberts MM, Haylock DN, et al. Comparison of haematological recovery times and supportive care requirements of autologous recovery phase peripheral blood stem cell transplants, autologous bone marrow transplants and allogeneic bone marrow transplants. Bone Marrow Transplant 1992; 9:277-84.
10. Siena S, Bregni M, Di Nicola M, et al. Durability of hematopoiesis following autografting with peripheral blood hematopoietic progenitors. Ann Oncol 1994; 5:935-41.
11. Civin CI, Gore SD. Antigenic analysis of hematopoiesis: a

review. J Hematother 1993; 2:137-44.
12. Wickenhauser C, Thiele J, Drebber U, et al. CD34+ human hematopoietic progenitor cells of the bone marrow differ from those of the peripheral blood: an immunocytochemical and morphometric study. Acta Haematol 1995; 93:83-90.
13. Carlo-Stella C, Cazzola M, De Fabritiis P, et al. CD34-positive cells: biology and clinical relevance. Haematologica 1995; 80:367-87.
14. Tjonnfjord GE, Steen R, Evensen SA, Thorsby E, Egeland T. Characterization of CD34+ peripheral blood cells from healthy adults mobilized by recombinant human granulocyte colony-stimulating factor. Blood 1994; 84:2795-801.
15. Bender JG, Unverzagt KL, Walker DE, et al. Identification and comparison of CD34-positive cells and their subpopulations from normal peripheral blood and bone marrow using multicolor flow cytometry. Blood 1991; 77:2591-6.
16. To LB, Haylock DN, Dowse T, et al. A comparative study of the phenotype and proliferative capacity of peripheral blood (PB) CD34+ cells mobilized by four different protocols and those of steady-phase PB and bone marrow CD34+ cells. Blood 1994; 84:2930-9.
17. Siena S, Bregni M, Bonsi L, et al. Increase in peripheral blood megakaryocyte progenitors following cancer therapy with high-dose cyclophosphamide and hematopoietic growth factors. Exp Hematol 1993; 21:1583-90.
18. Tarella C, Benedetti G, Caracciolo D, et al. Both early and committed haemopoietic progenitors are more frequent in peripheral blood than in bone marrow during mobilization induced by high-dose chemotherapy + G-CSF. Br J Haematol 1995; 91:535-43.
19. Fritsch G, Stimpfl M, Kurz M, et al. The composition of CD34 subpopulations differs between bone marrow, blood and cord blood. Bone Marrow Transplant 1996; 17:169-78.
20. Terstappen LWMM, Huang S, Safford M, Lansdorp PM, Loken MR. Sequential generations of hematopoietic colonies derived from single non lineage-committed CD34+ CD38- progenitor cells. Blood 1991; 77:1218-27.
21. Huang S, Terstappen LWMM. Formation of hematopoietic microenvironment and hematopoietic stem cells from single human bone marrow stem cells. Nature 1992; 360:745-9.
22. Huang S, Terstappen LWMM. Lymphoid and myeloid differentiation of single human CD34+, HLA-DR+, CD38- hematopoietic stem cells. Blood 1994; 83:1515-26.
23. Berardi AC, Wang A, Levine JD, Lopez P, Scadden D. Functional isolation and characterization of human hematopoietic stem cells. Science 1995; 267:104-8.
24. Rusten LS, Jacobsen SEW, Kaalhus O, Veiby OP, Funderud S, Sheland EB. Functional differences between CD38- and DR- subfractions of CD34+ bone marrow cells. Blood 1994; 84:1473-81.
25. Apperley JF. Umbilical cord blood progenitor cell transplantation. Bone Marrow Transplant 1994; 14:187-96.
26. Steen R, Tjonnfjord GE, Egeland T. Comparison of the phenotype and clonogenicity of normal CD34+ cells from umbilical cord blood, granulocyte colony-stimulating factor-mobilized peripheral blood, and adult bone marrow. J Hematother 1994; 3:253-62.
27. D'Arena G, Cascavilla N, Di Giorgio G, Carotenuto M. Flow cytometric analysis of CD34+ cord blood cells. (Abstract). Eur J Histochem 1995; 39 (Suppl. 3):41.
28. Almici C, Carlo-Stella C, Wagner JE, Rizzoli V. Umbilical cord blood as a source of hematopoietic stem cells: from research to clinical application. Haematologica 1995; 80:473-9.
29. Tong J, Gianni AM, Siena S, et al. Primitive hematopoietic progenitor cells are present in peripheral blood autografts. Blood Cells 1994; 20:351-63.
30. Tong J, Hoffman R, Siena S, et al. Characterization and quantitation of primitive hematopoietic progenitor cells present in peripheral blood autografts. Exp Hematol 1994; 22:1016-24.
31. Loken MR, Shaw VO, Dattilio KL, Civin CI. Flow cytometric analysis of human bone marrow. I. Normal erythroid development. Blood 1987; 69:255-63.
32. Thomas ML. The leukocyte common antigen family. Ann Rev Immunol 1989; 7:339-69.
33. Craig W, Poppema S, Little MT, Dragowska W, Lansdorpo P. CD45 isoform expression on human hemopoietic cells at different stages of development. Br J Hematol 1994; 88:24- 30.
34. Brandt J, Briddell RA, Srour EF, Leemhuis TB, Hoffman R. Role for c-kit ligand in the expansion of human hematopoietic progenitor cells. Blood 1992; 79:634-41.
35. Keller JR, Ortiz M, Ruscetti FW. Steel factor (c-kit ligand) promotes the survival of hematopoietic stem/progenitor cells in the absence of cell division. Blood 1995; 86:1757-64.
36. Ogawa M. Differentiation and proliferation of hematopoietic stem cells. Blood 1993; 81:2844-53.
37. Papayannopoulou T, Brice M, Broudy VC, Zsebo KM. Isolation of c-kit receptor-expressing cells from bone marrow, peripheral blood, and fetal liver: functional properties and composite antigenic profile. Blood 1991; 78:1403-12.