Kinetics and immunophenotypic characterization of circulating hematopoietic progenitor cells after peripheral blood stem cell transplantation

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A B S T R A C T

Background and Objectives. Hematopoietic progenitor cells (HPC) circulate in the peripheral blood (PB) before and after engraftment following autologous or allogeneic peripheral blood stem cell transplantation (PBSCT), although the characteristics of these cells are not known. CD34 protein is a reliable marker for identifying the fraction of hematopoietic cells in which HPC are contained. The $CD34^+$ cells represent a heterogeneous cell population consisting of both primitive uncommitted as well as pluripotent committed progenitors. The aim of this study was to investigate the kinetics and immunophenotypic characteristics of these post-transplant circulating progenitor cells.

Design and Methods. Forty-seven auto-PBSCT and nine allo-PBSCT recipients were selected for this study. Samples of PB were taken from each patient 4, 9, 11, 14, 16 and 18 days after the transplant. Cells were incubated with the following combinations of monoclonal antibodies: CD34-FITC/CD90-PE/CD38-CyCrome; CD34-FITC/CD117-PE/HLA-DR-PerCP; CD34-FITC/CD13-PE/CD33-CyCrome and the cells were then analyzed by flow cytometry.

Results. $CD34^+$ cells were undetectable on day +4; they reappeared from day +9 to day +18 along with neutrophil and platelet recovery. Subsets of $CD34^+$ HPC enriched in pluripotent stem cells ($CD90^+/CD38^{low}$ or $HLADR^-$) were hardly detected during the very early post-transplant period. HPC that expressed myeloid associated antigens (CD33, CD13, and CD117) increased after engraftment and constituted the largest proportion of the hematopoietic progenitor cells.

Interpretation and Conclusions. Circulating HPC could be detected in the early period after PBSCT. The qualitative and quantitative composition of these cells is similar to that found among HPC from mobilized PB.

Key words: immunophenotypic hematopoietic progenitor cells, CD34 subsets.

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Autologous and allogeneic peripheral blood stem cell transplantations (PBSCT) have been demonstrated to provide rapid hematologic reconstitution after marrow-ablative chemotherapy use to treat hematologic or other malignant neoplasms.[1-3] In contrast to animal studies in which the reconstitution of hematopoiesis after transplantation has been extensively studied, little is known about the reconstitution of normal hematopoiesis in humans.

Hematopoietic stem or progenitor cells (HPC) circulate in the peripheral blood (PB) after auto or allo-PBSCT,[4-6] but their characteristics are not well known. Since monoclonal antibodies and nucleic acid-based probes for HPC-associated proteins have become available in the last decade, a more comprehensive characterization of HPC can be made.

At present, it is known that the CD34 protein is a reliable marker for identifying a small fraction of bone marrow and peripheral blood mononuclear cells in which HPC are contained. The $CD34^+$ cells represent a heterogeneous cell population consisting of primitive uncommitted and pluripotent progenitors as well as committed stem cells.[7] The most primitive hematopoietic stem cells have been shown to lack expression of the CD38 antigen, whereas more differentiated committed progenitors express both CD34 and CD38 antigens as well as other lineage markers.[8-10]

The aim of this study was to investigate the kinetics and immunophenotypic characteristics of these post-transplant circulating progenitor cells.

Design and Methods

Patients

Forty-seven auto-PBSCT and nine allo-PBSCT recipients were consecutively evaluated from January 2000 to August 2002 for

this study. Their median age was 46.5 years (17-69). Twenty patients had non-Hodgkin's lymphoma, 16 myeloma, 8 Hodgkin's lymphoma, 7 acute leukemia, 4 solid neoplasms and 1 patient chronic myeloid leukemia. The characteristics of these auto and allo-PBSCT receipients are listed in Tables 1 and 2. Granulocyte colony-stimulating factor (G-CSF) was given routinely after auto-PBSCT (5 mg/kg/day) from day +5 until neutrophil recovery.

Rapid hematologic recovery was observed in 54 of the 56 patients after PBSCT. One autologous transplant recipient and one allogeneic recipient reached sustained myeloid engraftment only at days 23 and 25, respectively, after transplantation; both also had delayed platelet recovery. The delayed recovery was correlated with HLA disparity in allo-PBSCT[11] and with a suboptimal dose of $CD34^+$ cells reinfused in the autologous transplant.[12] Both patients were excluded from the analysis.

Cell preparation

Twenty milliliter samples of peripheral blood in heparin were obtained on days 4, 9, 11, 14, 16 and 18 after PBSCT. Peripheral blood mononuclear cells (PBMC) were isolated using the Ficoll-Hypaque method. The PBMC was mixed with a dimethyl sulfoxide (DMSO) solution (Sigma, St Louis, USA), used at a final concentration of 10% (vol/vol). The final volumes of PBMC were transferred to two cryostorage vials (Nunc Cryosystem Vials, Nunc A/S, Roskilde, Denmark) and stored at –80°C in a freezer.

The PBMC were thawed using Rubinstein's method[13] which minimizes DMSO toxicity by reducing the osmolarity in the cell suspension under controlled conditions. This method provides almost total recovery of viable hematopoietic progenitor cells.

Flow cytometric analysis

The contents of the vials were used, after thawing, to study the cells' surface marker's. Cells were incubated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridin- chlorophyll-A-protein (PerCP) or CyCrome-conjugated monoclonal antibodies (MoAb) using the following combinations: (i) anti CD34-FITC (Becton Dickinson [BD], San José, CA, USA)/anti CD90-PE (BD)/anti CD38-CyCrome (BD); (ii) CD34-FITC (BD)/CD117-PE (Caltag Laboratories, Burlingame, CA, USA)/HLA-DR-PerCP (BD); (iii) CD34-FITC (BD)/CD13-PE (BD)/CD33-CyCrome (BD). The IgG_1-FITC (BD)/IgG_1-PE (BD)/anti CD45-PerCP (BD) combinations of MoAb were used as negative controls.

Because we performed the immunophenotypic study on cryopreserved samples and this procedure may alter some antigens,[14] we determined the same antigens that we investigated in those samples on fresh and cryopreserved cells of cord blood and PB-mobilized and fresh samples of hematopoietic progenitors circulating after transplantation.

Samples were analyzed by a FACscan flow cytometer (BD), equipped with Cell Quest and Paint-A-Gate software (BD). The analysis consisted of three sequential steps (Figure 1): first we collected 5×10^3 cells to detect a cluster of $CD34^+$ cells in a SSC versus CD34 two-parameter plot; second, $CD34^+$ cells were selected from among the leukocytes; and third, an analysis was run to detect specific markers. This method allows the percentage of each $CD34^+$ cell subset to be calculated within the whole $CD34^+$ cell population. The third step was done only when a minimum of 10^3 $CD34^+$ cells was acquired because, as other authors pointed out, we need at least 100 cells to form a cluster for accurate evaluation of rare populations.[15]

Statistical analysis

The Statistical Package for the Social Sciences (SPSS) was used to compute the estimates. The following prognostic factors were analyzed: sex (male versus female), age, diagnosis, cycles before transplantation, type of mobilization (chemotherapy plus hematopoietic growth factor [HGF] versus HGF alone), type of transplantation (auto or allo-PBSCT), dose of infused $CD34^+$ cells and dose of infused CFU-GM cells. The influence of these prognostic factors on the number of circulating HPC was performed using Spearman's rank correlation coefficients for univariate analyses and a multiple regression model for multivariate analyses.

Results

$CD34^+$ cells were undetectable on day 4 after transplantation. A $CD34^+$ population reappeared between day 9 and day 18 after transplantation, depending on the patient, contemporaneously with neutrophil and platelet recovery.

On day 9 after transplantation, 11 patients (20.37%) show reactivity to the CD34 marker. Only one of these 11 patients (patient #7) had $\geq$ 1000 $CD34^+$ cells, so no other markers were analyzed at that point. On day 11 we detected $CD34^+$ cells in 33 patients (61.11%); the overall mean percentage of $CD34^+$ cells in the samples analyzed was 0.24% (SD$\pm$0.37), and we explored reactivity with the other markers in 20 patients. The mean percentage of $CD34^+$ cells on day 14 was 0.33% (SD$\pm$0.30) and these cells were detectable in 44 patients (78.6%); subpopulations were studied in 33 patients. On days 16 and 18 almost all the patients had a $CD34^+$ population (90.4% on day 16 and 85% on day 18). The mean percentage of $CD34^+$ cells detected was 0.28% (SD $\pm$ 0.62) and 0.25% (SD $\pm$ 0.43) on day 16 and day 18, respectively. We could study subsets of

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Table 1. Characteristics of the patients undergoing autologous PBSCT.

Patient	*Diagnosis*	*Status at transplant*	*Previous cycles*	*Type of mobilization*	*Conditioning regimen*	*N. infused CD34+ cells (×10⁶/kg)*	*N. infused CFU-GM (×10⁴/kg)*	*Days to granulocytes >500*	*Days to PL >20000*
1	Myeloma	CR	4	G-CSF	melphalan 200	2.99	4.75	11	12
2	NHL	CR	7	G-CSF	BEAM	3.60	6.19	13	14
4	Myeloma	PR	4	G-CSF	melphalan 200	3.28	5.11	14	11
5	HD	CR	6	G-CSF	BEAM	3.22	5.23	15	11
7	HD	CR	6	G-CSF	BEAM	4.82	8.44	11	13
8	AML	CR	4	G-CSF	BUCY	9.60	13.65	17	13
9	NHL	CR	12	cyclophosphamide	BEAM	1.71	2.78	16	11
13	AML	CR	4	G-CSF	BUCY	5.81	9.76	18	11
14	HD	CR	9	G-CSF	BEAM	5.80	8.98	15	10
15	Testicular ca	PR	8	IFE	CEI	3.05	5.64	11	12
16	Testicular ca	PR	9	IFE	CEI	2.55	4.50	11	16
17	NHL	CR	6	cyclophosphamide	BEAM	4.03	7.06	13	10
18	NHL	CR	10	G-CSF	BEAM	4.34	7.71	14	10
19	Myeloma	PR	8	cyclophosphamide	melphalan 200	6.83	11.15	13	9
21	NHL	CR	10	cyclophosphamide	BEAM	3.72	6.48	14	9
22	HD	CR	12	G-CSF	BEAM	2.89	4.83	13	11
23	Myeloma	PR	6	G-CSF	melphalan 200	2.92	4.99	13	12
25	Myeloma	PR	8	cyclophosphamide	CBV	6.82	11.16	14	9
26	NHL	CR	4	mega-CHOP	BEAM	2.73	ND	13	11
27	Myeloma	PR	6	cyclophosphamide	melphalan 200	2.98	5.36	15	11
28	ALL	CR	4	cyclophosphamide	BUCY	17.60	32.57	13	10
29	Myeloma	PR	8	cyclophosphamide	melphalan 200	8.82	12.23	14	12
31	HD	AD	8	G-CSF	BEAM	3.20	5.63	17	11
32	HD	PR	8	mini-BEAM	BEAM	4.21	6.95	13	9
34	Myeloma	PR	6	G-CSF	melphalan 200	3.37	5.99	17	11
35	Myeloma	AD	16	cyclophosphamide	melphalan 200	2.20	3.49	11	17
36	NHL	CR	5	mini-BEAM	BEAM	15.90	ND	9	10
37	Breast ca	PR	6	G-CSF	CTCb	3.65	6.25	9	9
38	NHL	CR	20	G-CSF	BEAM	1.89	3.11	21	192
39	NHL	CR	4	cyclophosphamide	BEAM	1.52	3.11	13	14
40	NHL	CR	5	cyclophosphamide	BEAM	8.47	12.72	11	10
41	Breast ca	PR	6	G-CSF	CTCb	3.86	7.45	10	9
42	Myeloma	PR	4	G-CSF	melphalan 200	3.93	5.40	10	11
43	NHL	CR	5	mega-CHOP	BEAM	5.37	9.66	11	12
44	NHL	CR	4	cyclophosphamide	BEAM	2.31	4.18	10	11
45	Myeloma	PR	4	G-CSF	melphalan 200	11.9	16.8	13	9
46	NHL	CR	4	G-CSF	BEAM	3.03	5.46	13	11
47	NHL	CR	5	mini-BEAM	BEAM	5.27	8.96	10	9
48	NHL	CR	4	G-CSF	BEAM	8.12	9.12	11	9
49	Myeloma	PR	7	G-CSF	CBV	11.90	16.80	10	15
50	Myeloma	PR	6	cyclophosphamide	melphalan 200	6.83	11.15	14	15
51	Myeloma	PR	8	G-CSF	melphalan 200	3.72	6.77	11	10
52	Myeloma	PR	6	G-CSF	melphalan 200	2.33	4.05	10	12
53	NHL	PR	7	G-CSF	BEAM	8.18	14.72	15	11
54	NHL	CR	12	SCF	BEAM	2.37	3.81	13	18
55	NHL	CR	10	G-CSF	BEAM	2.16	3.67	10	11
56	NHL	CR	16	cyclophosphamide	BEAM	2.63	ND	13	12

PL: platelets; NHL: non-Hodgkin's lymphoma; HD: Hodgkin's disease; AML: acute myeloblastic leukemia; ALL: acute lymphoblastic leukemia; CR: complete response; PR: partial response; AD: active disease; IFE: ifosfamide, etoposide; CHOP: cyclophosphamide, doxorubicin, vincristine, prednisone; BEAM: carmustine, etoposide, cytarabine, melphalan; BUCY: busulfan, cyclophosphamide; CEI: carboplatin, etoposide, ifosfamide; CBV: cyclophosphamide, carmustine, etoposide; CTCb: cyclophosfamide, thiotepa, carboplatin.

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CD34⁺ cells in 35 patients (62.4%) on day 16 and in 28 (49.8%) on day 18. Table 3 shows the reactivity of CD34⁺ cells with the other markers examined in this study. Most of the CD34⁺ cells co-expressed CD33, CD13, and CD117 antigens. By contrast a small proportion of CD34⁺ cells failed to co-express HLA-DR or co-expressed CD90/CD38^{low}. Subsets of CD34⁺ HPC enriched in pluripotent stem cells were hardly detect-

Table 2. Characteristics of the patients undergoing allogeneic PBSCT.

Patient	*Diagnosis*	*Status at transplant*	*Previous cycles*	*Type of mobilization*	*Conditioning regimen*	*N. infused CD34⁺ cells (×10⁶/kg)*	*N. infused CFU-GM (×10⁴/kg)*	*Days to granulocytes >500*	*Days to PL >20000*
3	CML	1st chronic phase	24	G-CSF	BUCY	3.81	5.23	23	186
6	HD	CR	10	G-CSF	FLU-MEL	4.02	9.49	16	11
10	Myeloma	PR	10	G-CSF	FLU-MEL	2.01	3.00	17	11
11	AML	CR	3	G-CSF	BUCY	10.48	19.20	13	10
12	AML	CR	4	G-CSF	BUCY	9.70	12.30	15	9
20	HD	CR	10	G-CSF	FLU-MEL	3.92	6.12	18	12
24	AML	CR	3	G-CSF	BUCY	4.83	7.49	17	11
30	AML	CR	3	G-CSF	BUCY	6.47	9.23	13	11
33	NHL	PR	11	G-CSF	FLU-MEL	6.83	10.43	10	10

PL: platelets; CML: chronic myeloid leukemia; HD: Hodgkin's disease; AML: acute myeloblastic leukemia; NHL: non-Hodgkin's lymphoma; CR: complete response; PR: partial response; BUCY: busulfan, cyclophosphamide; FLU-MEL: fludarabine, melphalan.

ed during the very early post-transplant period. These HPC diminished on days 14 to 18, whereas HPC that expressed pan-myeloid associated antigens (CD33, CD13, and CD117) increased after engraftment and came to constitute the majority of the hematopoietic progenitor cells. The number of circulating CD34⁺ cells after transplantation was found to be significantly influenced by the following factors in the univariate analysis: cycles before transplantation ($p<0.01$), type of transplantation –auto or allo-PBSCT- ($p<0.001$), dose of infused CD34⁺ cells ($p<0.001$) and dose of infused CFU-GM cells ($p<0.05$) (Table 4). In the multivariate analysis only the type of transplantation and the dose of infused CD34⁺ cells were significant factors (Table 5).

In the subgroup analyses, great differences were observed between percentages of total CD34⁺cells and subpopulations of CD34 in autologous and allogeneic

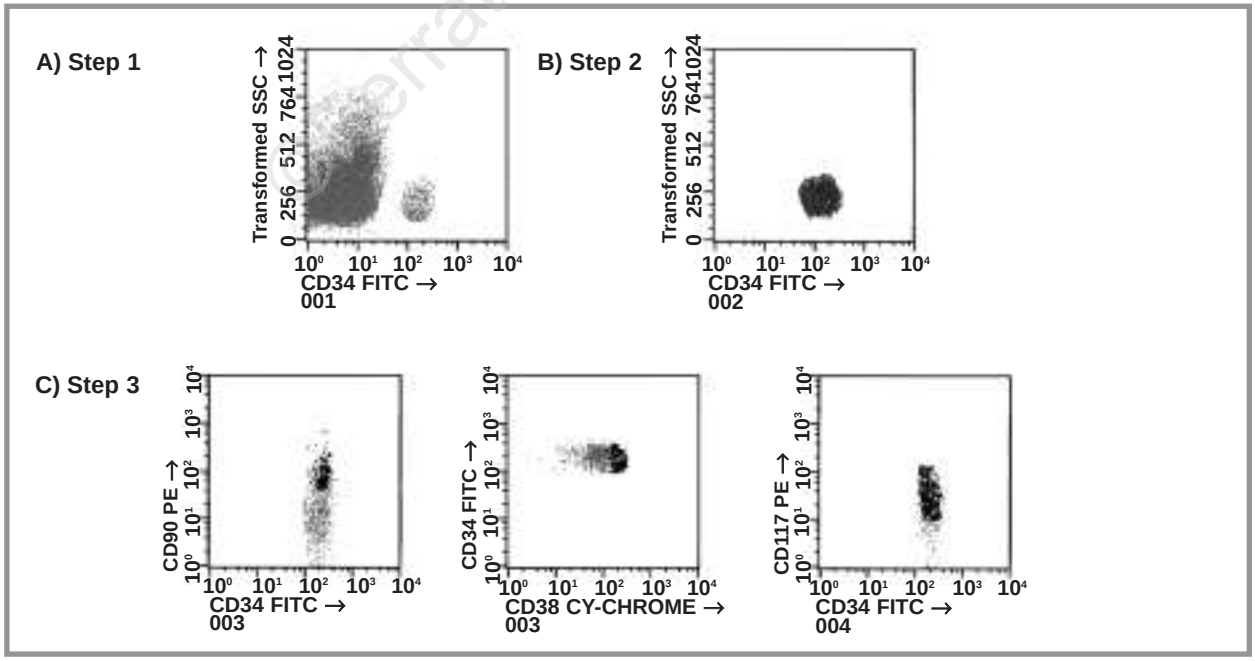

Figure 1. Characterization of CD34⁺ cell subsets.

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Table 3. Percentage of CD34⁺ cell subpopulations in peripheral blood after PBSCT. The analysis was based on triple markers.

	Days			
Percentage	*+11*	*+14*	*+16*	*+18*
CD34⁺/CD90⁺/CD38^{low}	10.26±8.69	6.71±5.71	4.31±4.18	3.10±2.18
CD34⁺/HLA-DR⁻	3.05±2.85	1.49±1.74	0.36±0.33	0.40±0.38
CD34⁺/CD117⁺	66.39±10.06	70.37±9.20	72.28±9.90	76.63±10.93
CD34⁺/CD13⁺	89.69±2.41	92.43±4.11	94.14±3.22	94.78±3.44
CD34⁺/CD33⁺	92.82±2.01	95.07±3.06	96.37±2.48	97.39±1.92

Table 4. Factors affecting the number of circulating CD34⁺ cells after transplantation: univariate analyses.

	Spearman's coefficient	*P value*
Sex	0.087	0.43
Age	0.139	0.19
Diagnosis	0.124	0.21
Cycles before transplantation	0.408	0.01
Type of mobilization	0.190	0.56
Type of transplantation	0.778	0.001
Dose of infused CD34⁺ cells	0.741	0.001
Dose of infused CFU-GM	0.280	0.05

Table 5. Factors affecting number of circulating CD34⁺ cells after transplantation: multivariate analyses.

	R multiple	*P value*
Cycles before transplantation	0.332	0.16
Type of transplantation	0.701	0.01
Dose of infused CD34⁺ cells	0.877	0.01
Dose of infused CFU-GM	0.493	0.12

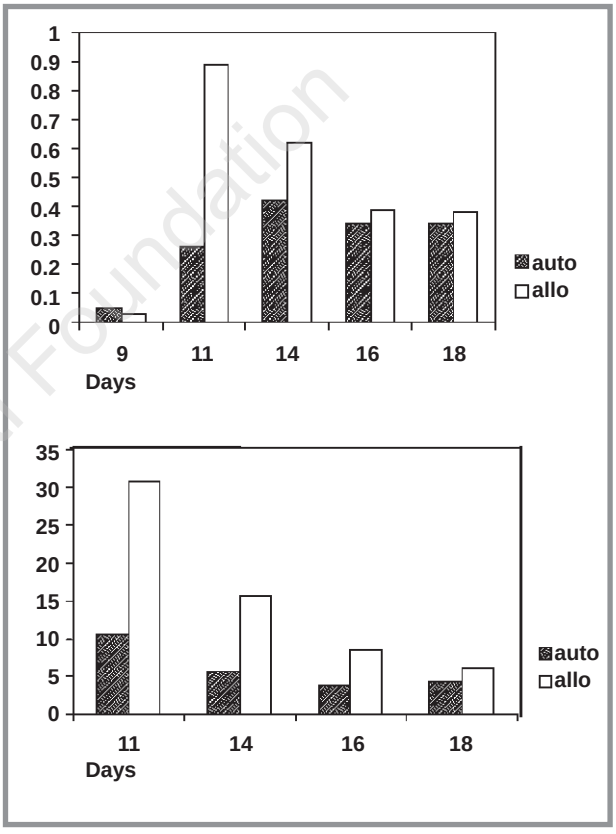

Figure 2. Percentages of total CD34⁺ cells and primitive HPC in auto and allo-PBSCT.

transplant recipients (Table 6). Compared with auto-PBSCT, allo-PBSCT contained a greater number of total CD34⁺ cells and more immature CD34⁺ HPC expressing CD90 or without expression of HLA-DR. There were significant differences in the percentages of these populations in autologous and allogeneic transplant recipients (Figure 2). It should be noted that the percentage of CD90⁺ cells was three times smaller in auto-PBSCT recipients than in allogeneneic recipients on day 11. There was no significant difference in the number of committed myeloid populations (CD33⁺, CD13⁺, and CD117⁺) between groups.

Discussion

Hematopoietic progenitor cells circulate in the peripheral blood after auto or allo-PBSCT.[4-6] The combination of flow cytometry and multiple staining with monoclonal antibodies has allowed HPC to be characterized better.[8-10]

In this study, we investigated the kinetics and immunophenotypic characteristics of HPC circulating in peripheral blood during the early stages after PBSCT. Our results clearly confirm that HPC could be detected in the post-transplant period (day +9 until +18).

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Table 6. Comparison of percentages of total CD34 and subpopulations of CD34 in auto and allo-PBSCT.

	Autologous				*Allogeneic*			
	Days							
Percentage	*+11*	*+14*	*+16*	*+18*	*+11*	*+14*	*+16*	*+18*
$CD34^+$	0.26±0.17	0.42±0.28	0.34±0.17	0.34±0.12	0.89±0.52	0.62±0.43	0.39±0.19	0.38±0.15
$CD34^+/CD90^+/CD38^{low}$	10.54±8.26	5.70±5.92	3.78±3.08	4.22±2.60	30.7±12.23	15.65±8.36	8.56±3.31	6.06±2.68
$CD34^+/HLA^-DR^-$	2.25±2.12	1.00±0.82	0.11±0.10	0.37±0.32	9.23±6.33	1.45±0.62	0.00	0.00
$CD34^+/CD117^+$	64.83±10.72	71.78±9.53	71.55±9.44	78.59±10.11	69.86±9.60	78.40±8.78	84.79±7.46	74.70±7.82
$CD34^+/CD13^+$	89.69±3.61	94.71±4.28	95.41±3.67	96.13±3.34	91.65±5.23	93.46±3.27	88.35±3.42	88.10±3.22
$CD34^+/CD33^+$	92.57±2.82	96.85±3.94	97.46±2.91	98.14±1.89	92.45±4.78	93.46±4.22	91.23±3.62	96.44±4.11

We did not investigate the kinetics of progenitor cells on day +1 in blood as Mahmut *et al.* had done,[6] but otherwise our data concerning the reappearance of HPC along with neutrophil and platelet recovery do not differ from those reported data. It is known that $CD34^+$ HPC constitute a heterogeneous population of cells including a small proportion of pluripotent stem cells and a much larger population of precursors, which are already committed to the different hematopoietic cell lineages.[10] Uncommitted progenitors that can repopulate bone marrow after transplantation are $CD34^+$ cells which do not express, or express very low levels of CD38 and HLA-DR.[9,10,16] $CD34^+CD90^+$ and $CD34^+CD117^+$ cells identify a much larger proportion of the $CD34^+$ population of which a relatively small percentage would correspond to uncommitted pluripotent HPC.[17] In contrast, more mature progenitors co-express CD34 antigen with the lineage-specific antigens.[10,16]

To the best of our knowledge, no previously published study has compared the frequencies of $CD34^+$ cell subsets in circulating HPC from individuals after PBSCT.

The phenotypic profile of $CD34^+$ cells circulating in PB after stem cell transplantation showed that a small percentage of these cells were the most primitive hematopoietic progenitor cells, $CD34^+$ HLA^-DR^-, whereas the much larger proportion of $CD34^+$ cells included relatively scanty pluripotent HPC ($CD34^+$ $CD90^+$ $CD38^{low}$ or $CD34^+$ $CD117^+$) and a higher percentage of myeloid-committed subsets (CD33 or $CD13^+$).

Lanza *et al.* showed that class I CD34 epitopes were reduced in samples after freezing with DMSO, whereas class II and class III epitopes were better preserved. Nevertheless, a significant increase in the antibody binding capacity of cryopreserved CD34 cells that stained with CD34 epitope class II and class III reactive MoAbs was also documented.[14] Others authors reported effects on cell subset proportions and fluorescence intensity in cryopreserved samples (most of which were peripheral blood samples) and noted small changes after thawing, but of no practical importance.[18,19]

Since we used a monoclonal antibody directed against a class II epitope in our analyses, it is possible that we overestimated the percentage of CD34 cells, and others antigens. We analyzed fresh and cryopreserved samples of cord blood and mobilized PB; our findings suggest that freezing is unlikey to have caused a selective loss of particular subsets or fluorescence intensity.

Previous studies have shown the phenotype of HPC in other compartments, such as bone marrow (BM), mobilized PB and umbilical cord blood (UCB).[9,10,20-22] Interestingly, the frequencies of circulating $CD34^+$ cells, in either mobilized PB or UCB with myeloid associated molecules (CD33 and CD13), were comparable to those found in our study, with means of about 92-97% and 89-94%, respectively, but they represent a greater fraction than in adult bone marrow,[10,20,21] just as we found for cells co-expressing CD34 and CD90 antigens. Nevertheless, a higher proportion of $CD34^+$ HLA^-DR^- progenitor cells was found among $CD34^+$ HPC from UCB than from BM, mobilized PB and circulating HPC after transplantation, in which this subset is usually present at similar frequencies (means 0.3-3%).[9,10,20] This suggests that the number of $CD34^+$ cells infused, harvested after PB mobilization, does affect the numbers of progenitors after transplantation.

There was a significantly higher percentage of circulating $CD34^+$ cells after allogeneic than after autologous PBSCT; this further supports the relationship between amount of CD34 infused and CD34 detected after transplantation. It should be pointed out that standardized methods for assessing $CD34^+$ HPC subsets have not yet been implemented, which might explain important differences between results in different reports.

Lowenthal *et al.*, Weaver *et al.* and other authors had observed the importance of CD34 cell dose infused for

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neutrophil and platelet recovery.[23,24] There is, therefore, reason to believe that circulating HPC and speed of neutrophil and platelet recovery could be related. We are currently investigating this topic. We conclude that circulating HPC could be detected in the early period after PBSCT and that the qualitative and quantitative composition of these cells is similar to that found among HPC from mobilized PB.

CAIb: conception, design, analysis and interpretation of data, drafting and revising the article and final approval of the version to be published; JdlF: conception and design, revising the article and final approval of the version to be published; CAr: analysis and interpretation of data, drafting and critically revising the article and final approval of the version to be published; CAlo: analysis and interpretation of data, drafting the article and approval of the final version to be published; EF: analysis and interpretation of data, drafting the article and approval of the final version to be published. The order of authorship was decided by authors themselves.

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