Haematologica 2000; 85:396-402
original paper

Transplantation & Cell Therapy

Relationhips between total CD34+ cells reinfused, CD34+ subsets and engraftment kinetics in breast cancer patients

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ABSTRACT

Background and Objectives. **The aim of the present study was to evaluate the correlation between the number of CD34+ cells transfused and the duration of hypoplasia, and the relationship between various CD34+ subsets (CD34+/33–; CD34+/38-; CD34+/HLA-DR–; CD34+/Thy-1+) and engraftment kinetics in a series of patients with breast cancer treated with high doses of thiotepa and melphalan.**

Design and Methods. **We treated 42 consecutive patients: 19 in an adjuvant context (≥ 4 positive axillary nodes) and 23 for metastatic disease. A combination of thiotepa 600 mg/m² and melphalan 140-160 mg/m² was administered as the conditioning regimen. All patients received peripheral blood progenitor cells (PBPC) and growth factors for hematopoietic rescue.**

Results**. In univariate analysis, we found a significant relationship between the number of CD34+ cells reinfused and the time to hematologic recovery and the duration of hospital stay. We observed an inverse correlation between the number of CD34+ cells reinfused and the units of platelets transfused. Cox multivariate analysis confirmed that the number of CD34+ cells reinfused is the most effective predictor of time to hematologic recovery. CFU-GM resulted to be a better predictor of the duration of hospitalization.**

Interpretation and Conclusions. **We found a significant relationship between the number of PBPC reinfused and the time to hematologic recovery after high doses of thiotepa and melphalan. In our experience, the numbers of subsets of CD34+ cells infused did not give compared additional information to that provided by the total number of CD34+ cells infused.**

Key words: PBPC, engraftment kinetics, breast cancer, high-dose chemotherapy, CD34+ cells subsets

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Breast cancer is the most common indication for autografting among solid tumors, as confirmed by the number of registered transplants in Europe in 1998.[1] Recent retrospective clinical studies and one randomized trial have clearly demonstrated that recovery of hematopoiesis occurs significantly faster after reinfusion of peripheral blood progenitor cells (PBPC) than after autologous bone marrow transplantation (ABMT).[2-4] Furthermore, patients rescued with PBPC stayed in hospital a shorter time, had fewer febrile days, lower antibiotic use, and required fewer red cell and platelet transfusions than those receiving autologous bone marrow transplantation (ABMT).[2-4] Moreover, the use of PBPC may reduce the risk of tumor cell contamination.[5,6] The greater safety of PBPC is also reflected in its lower procedure-related mortality. In Europe PBPC have almost entirely replaced ABMT as hematopoietic support.[7] Flow cytometric determination of the subset of peripheral blood cells expressing the CD34 antigen is commonly used today to assess the progenitor cell content and correlates quite well with CFU-GM quantities.[8-10] Furthermore, the CD34+ cell count has proved to be a good predictor of engraftment kinetics, especially as far as platelet recovery is concerned.[11-13] A major clinical issue is to define the minimum number of cells necessary for rapid hematopoietic recovery after high-dose chemotherapy, although some investigators have already proposed 2 or 2.5×10^6 cells/kg/bw as the minimal threshold for rapid hematopoietic reconstitution.[14,15] The optimal level of CD34+ cells to be reinfused was previously defined as the dose of PBPC that allows >95% of the patients to recover ≥ 500 white blood cells/μL by 11 days after transplantation and ≥ 50,000 platelets/μL within 14 days.[16] CD34+ cells are not a single population of cells, but represent a continuum of progenitors from the common stem cell to committed progenitors. We, therefore, wondered about the role of the various CD34 subsets in hematopoietic recovery. The aim of our study was to evaluate the correlation between the number of CD34+ cells transfused and time to hematologic recovery, transfusion needs, mucositis grade and the duration of hospital stay, in a series of patients with breast cancer receiving high doses of thiotepa and melphalan. Afterwards, we analyzed the relationship between engraftment kinetics and the various CD34 subsets.

Design and Methods

Patient characteristics

Forty-two consecutive breast cancer patients referred to our Oncology and Hematology Department for high dose chemotherapy were evaluated. Nineteen patients with more than four positive axillary nodes were treated in an adjuvant context, while 23 patients had metastatic disease. The characteristics of the patients are listed in Table 1.

Chemotherapy

High-dose cyclophosphamide (7 g/m^2) was employed as the anticancer mobilizing regimen in the adjuvant setting, while epirubicin 150 mg/m^2 was selected for metastatic disease, both administered with hematopoietic growth factors, according to the protocol in use at our institution at the time of the study. The conditioning regimen consisted of thiotepa 600 mg/m^2 on day –3 and melphalan on day –1 at a dose of 140 mg/m^2 in metastatic patients and 160 mg/m^2 in the adjuvant setting. All patients received PBPC as hematopoietic rescue on day 0. Hematopoietic growth factor (filgrastim 5 mg/kg/bw) administration started 24 hours after PBPC infusion and continued until absolute neutrophil count (ANC) was greater than 1,000/µL for three consecutive days.

PBPC collection

Blood samples were taken once a day starting on day 7 after the first day of mobilizing chemotherapy and, when the WBC count clearly began to rise, CD34+ cells were detected using 8G12 antibody. The decision to start leukapheresis was taken when the number of circulating CD34+ cells was ≥ 20/µL of peripheral blood. The number of CD34+ cells considered as the target for each high-dose chemotherapy was ≥ 2×10^6/kg/bw, as accepted by the majority of investigators.[6] The PBPC collection procedures were performed using the MNC program of Spectra COBE BCT, Apheresis System (Cobe, Lakewood, Co, USA). A total blood volume of 6-9 L per apheresis was processed through double lumen apheresis catheters or by venous puncture for 3-4 h at a flow rate of 45-50 mL/min. PBPC concentrates were cryopreserved with a final dimethyl sulfoxide concentration of 10%. Suspensions were frozen in cryopreservation bags and stored in liquid nitrogen. Granulocyte-macrophage colony-forming units (CFU-GM) were assayed in a semisolid medium, and after 14 days of incubation, all colonies with more than 40 cells were counted by means of an inverted microscope.

PBPC enumeration

The percentage of cells that expressed the CD34 antigen was determined in a sample of the leukapheresis products by direct immunofluorescence just before cryopreservation. For dual-color flow-cytometric analysis 1×10^6 cells were incubated at 4°C for 30 minutes each time with the anti-CD34 antibody and one of among the other antibodies we tested. We used the following panel of monoclonal antibodies (MoAb): CD34 (8G12 antibody) CD33, CD38 (all from Becton Dickinson, San José, CA, USA), Thy-1 (IMMUCOR, Milan, Italy), and HLADR (ORTHO, Milan, Italy). After lysis of the erythrocytes with isomolar ammonium chloride for 10 minutes, flow-cytometric analysis was performed with an absolute flow cytometer (ORTHO, Milan, Italy). An isotype-matched mouse Ig served as the control for each couple of monoclonal antibodies.

Statistical analysis

The statistical analysis was performed using SAS software. Spearman's test was used to assess the correlation between apheresis products, namely MNC, CFU-GM, total CD34+, CD34+/Thy-1+, CD34+/38–, CD34+/HLADR–, CD34+/33– cells, as well as the correlation between number of CD34+ cells and clinical end points (units of platelets and red blood cells transfused, days with fever >38°C, days with severe mucositis).

The univariate analysis was carried out using the product-limit method of Kaplan-Meier and compared using a log-rank test. Total CD34+ cells, CD34+/Thy-1+ cells, CD34+/38– cells, CD34+/HLADR– cells, CD34+/33– cells plus MNC and CFU-GM were evaluated as dichotomous variables according to the median value and compared with the time to unassisted neutrophil and platelet recovery and the duration of hospitalization. A backward stepwise Cox proportional hazards regression model was used for multivariate analysis.

Table 1. The characteristics of the 42 patients.

Median age	43 years (26-60)
Treatment setting:	
adjuvant	19
metastatic	23
Previous adjuvant treatment:	
CMF	6
E/CMF	2
EC	1
FEC	1
Metastatic site	
Liver	7
Lung	4
Lymph node	3
Bone	1
Uterus & lymph node	1
Uterus & bone	1
Liver & lung	1
Lung & bone	1
Lung & lymph node	1
Liver, bone & Lymph node	1
Liver, lung & CNS	1
Liver, lung & bone	1

CMF: cyclophosphamide 600 mg/m^2, methotrexate 40 mg/m^2, fluorouracil 600 mg/m^2 on days 1 and 8 q 28 days for 6 cycles. E/CMF: epirubicin 120 mg/m^2 q 21 days for 3 cycles followed by CMF for 6 cycles. EC: epirubicin 90 mg/m^2 and cyclophosphamide 600 mg/m^2 q 21 days for 4 cycles. FEC: fluorouracil 500 mg/m^2, epirubicin 60 mg/m^2, cyclophosphamide 500 mg/m^2 q 28 days for 6 cycles.

Table 2. Progenitor cell levels in leukapheresis samples.

	Median value	*Range*
$CD34^+ \times 10^6$/kg	7.95	1.6-24.8
$CD34^+/33^- \times 10^6$/kg	3.3	0-15.4
$CD34^+/38^- \times 10^6$/kg	0.043	0-1.52
$CD34^+/Thy^+ \times 10^6$/kg	0.38	0-2.86
$CD34^+/HLA\text{-}DR^- \times 10^6$/kg	0.38	0-4.6
MNC $\times 10^8$/kg	2.15	0.83-9.2
CFU-GM $\times 10^4$/kg	136.2	17.3-722.6

Results

PBPC collection

A single leukapheresis procedure was sufficient to collect the target number of $CD34^+$ cells in the vast majority of the patients (88%), while a double procedure was necessary in 5 patients (12%). Leukapheresis were performed between days 10 and 20 after the beginning of chemotherapy (median day 12). The median value of $CD34^+$ cells collected and reinfused was 7.95×10^6/kg/bw (range 1.6-24.8) considering the entire population of patients, and was 6×10^6/kg/bw for the metastatic patients and 8.1×10^6/kg/bw in the adjuvant setting. The relative amounts of each $CD34^+$ cell subsets are listed in Table 2.

Hematopoietic recovery

Patients required a median of 9 days (range 8-11) to reach an ANC greater than 500/μL; then all patients achieved an ANC greater than 1,000/μL within 24 hours. The median times to reach an unsupported platelet count greater than 20,000/μL and 50,000/μL were respectively 10 days (range 0-16) and 13 days (range 10-22).

Statistical correlation

The total number of apheresis product $CD34^+$ cells was strongly correlated with the numbers of CFU-GM (r=0.7, p=0.0001) (Figure 1), $CD34^+/33^-$ (r=0.5, p=0.0002) and $CD34^+/HLADR^-$ (r=0.5, p=0.001), and more weakly with $CD34^+/38^-$ (r=0.38, p=0.01) and $CD34^+/Thy\text{-}1^+$ (r=0.34, p=0.03). It did not correlate with MNC value (r=0.26, p=0.9). We found an inverse correlation between the number of $CD34^+$ cells reinfused and the units of platelet needing to be transfused (r = –0.3, p=0.05). We did not observe any correlation between subsets of $CD34^+$ cells and transfusional need. In the same way, we did not find any correlation between the number of PBPC reinfused and the days with either grade III-IV mucositis or with fever >38°C.

Univariate analysis

Using the median value as a cut-off and employing the Kaplan-Meier procedure, we found a statistically significant relationship between the number of $CD34^+$ cells reinfused and the time to achieve an ANC greater than 500/μL (p=0.0029) and 1,000/μL (p=0.0009), the time to achieve an unsupported platelet recovery to 20,000/μL (p=0.004) and to 50,000/μL (p=0.0007). Additionally, we observed a significant relationship between the number of $CD34^+$ cells and the duration of hospital stay (p=0.01) [Figure 2a-e]. Finally, we analyzed the relationships between various subsets of $CD34^+$ cells and time to hematologic recovery and duration of hospital stay: the results are summarized in Table 3.

Multivariate analysis

Cox multivariate analysis was performed to determine which factors, considered as dichotomous variables, more reliably predicted days to neutrophil and platelet recovery, and the duration of hospitalization. The results confirmed that the number of $CD34^+$ cells reinfused is the most effective predictor of time to neutrophil recovery and unsupported platelet recovery to 50,000/μL. CFU-GM resulted to be better than $CD34^+$

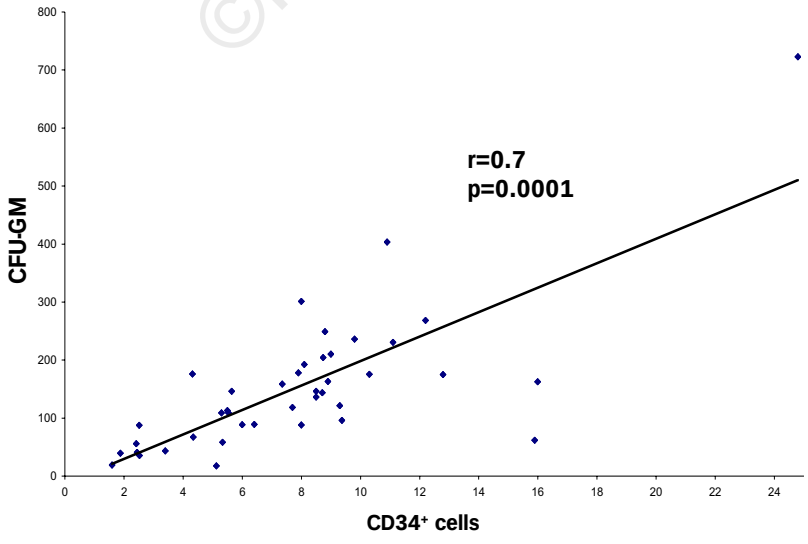

Figure 1. Correlation between total $CD34^+$ cell count ($x10^6$/kg/bw) and CFU-GM ($x10^4$/kg/bw).

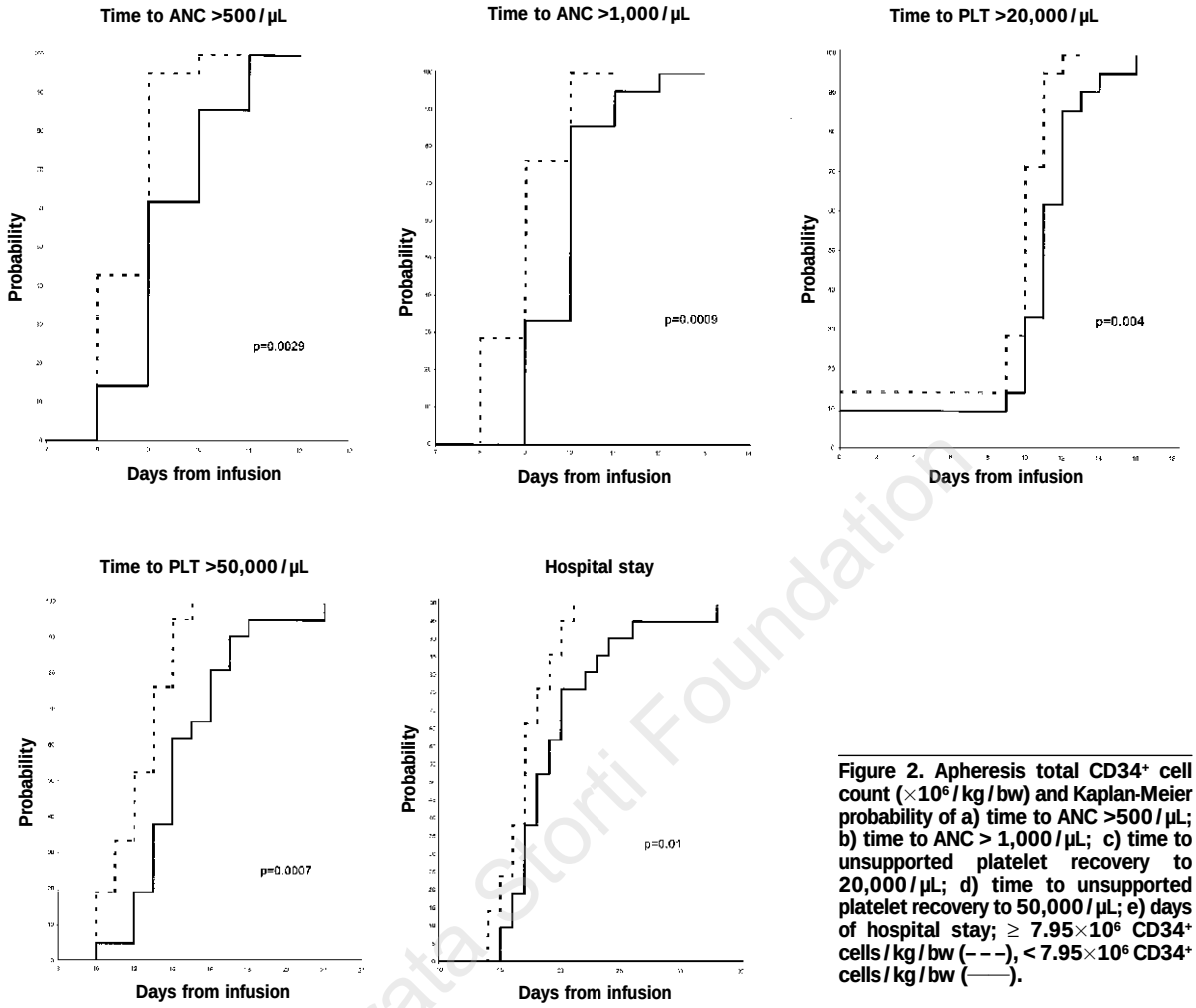

Figure 2. Apheresis total CD34+ cell count (×10⁶ / kg / bw) and Kaplan-Meier probability of a) time to ANC >500 / µL; b) time to ANC > 1,000 / µL; c) time to unsupported platelet recovery to 20,000 / µL; d) time to unsupported platelet recovery to 50,000 / µL; e) days of hospital stay; ≥ 7.95×10⁶ CD34+ cells / kg / bw (– – –), < 7.95×10⁶ CD34+ cells / kg / bw (——).

cell count as a predictor of time to platelet recovery up to 20,000/µL and duration of hospitalization. The number of CD34+/38- cells was found to be a positive predictor of time to platelet recovery to 50,000/µL, albeit a weaker one than CD34+ cells.

Subsequently, a second Cox multivariate analysis was performed comparing the various CD34+ cell subsets to hematopoietic reconstitution and the duration of hospital stay, considered this time as continuous variables. Total CD34+ cell number was found to be the most reliable predictive factor for time to hematologic recovery, while CFU-GM was again the most powerful predictor for the duration of hospitalization. The results of multivariate analyses are shown in detail in Table 4.

Discussion

The optimal number of CD34+ cells to be reinfused in patients undergoing peripheral blood progenitor cells transplantation after high-dose chemotherapy is still a matter of debate. Kiss *et al.* have already reported, in a small retrospective analysis in 17 patients with heterogeneous diseases, that the infused number of CD34 surface antigen-positive cells correlated with time to granulocyte and platelet recovery, and have shown the existence of a threshold-effect between rapid and slow engraftment when respectively more than or fewer than 5×10⁶ CD34+ cells/kg/bw are infused.[13] In a larger study including 692 heterogeneous patients, Weaver *et al.* too reported a clear dose-response relationship between the number of CD34+ cells reinfused and hematopoietic recovery, and defined an optimal CD34+ cell dose to be more than 5×10⁶/kg/bw.[17] More recently, Ketterer *et al.* demonstrated, in a retrospective analysis of 168 consecutive high-dose therapy regimens with PBPC rescue performed for lymphoproliferative diseases, that in multivariate analysis, the number of CD34+ cells reinfused was the only variable significantly correlated with neutrophil and platelet recovery.[18] Shpall *et al.* reviewed data from numerous studies and suggested that doses >5×10⁶ CD34+ cells/kg were associated with more

Table 3. Univariate analysis: significant results.

	Variable	*p value*
Time to ANC >500/µL	Total CD34$^+$	0.003
	CD34$^+$/33$^-$	0.009
	CD34$^+$/38$^-$	0.05
	CD34$^+$/Thy 1$^+$	0.05
	CFU-GM	0.0008
Time to ANC >1,000/µL	Total CD34$^+$	0.0009
	CD34$^+$/33$^-$	0.009
	CD34$^+$/Thy 1$^+$	0.02
	CFU-GM	0.003
Time to PLT >20,000/µL	Total CD34$^+$	0.004
	CD34$^+$/33$^-$	0.01
	CFU-GM	0.05
Time to PLT >50,000/µL	Total CD34$^+$	0.0007
	CD34$^+$/33$^-$	0.007
	CD34$^+$/38$^-$	0.01
	CD34$^+$/Thy 1$^+$	0.05
	CFU-GM	0.005
Hospital stay	Total CD34$^+$	0.01
	CD34$^+$/HLA-DR$^-$	0.004
	CFU-GM	0.005

rapid engraftment and lower probability of graft failure.[19] So, it would seem *the more, the better*. But all these studies considered groups of patients heterogenous as regard diseases, conditioning regimens or the number of transplants. This leaves the question open of whether recommended doses are suitable for all kinds of tumors, for all kinds of patients, and for all kinds of high-dose conditioning regimens. To assess this question we attempted to verify the relationship between the number of CD34$^+$ cells reinfused and engraftment kinetics in a homogeneous series of patients with breast cancer, treated with the same conditioning regimen containing high doses of thiotepa and melphalan. The result of this analysis demonstrated that also in our series of patients a higher number of CD34$^+$ cells reinfused was correlated with earlier hematopoietic engraftment as demonstrated by both neutrophil and unsupported platelet recovery. Moreover, a higher CD34$^+$ cell count shortened the duration of hospital stay and decreased the need for platelet transfusions. This might allow significant cost-saving, considering that a single leukapheresis procedure had been performed in the vast majority of patients (88%). The hypothesis that reinfusion of a larger number of CD34$^+$ cells could be cost-effective and contribute to the patient having a better quality of the life should be investigated in further studies. Mobilized blood stem cells, identified by the surface expression of CD34 antigen, may be further characterized by means of multiparameter flow cytometry to distinguish early stem and multipotent progenitor cells (CD34$^+$, Thy-1$^+$, CD38$^-$) from late unipotent progenitor cells committed to the myeloid lineage (CD34$^+$, Thy1$^-$, CD33$^+$). Therefore, we wondered about the role the various CD34$^+$ subsets, CFU-GM and MNC played in engraftment kinetics and investigated this by Cox proportional multivariate analysis. We did not find any CD34$^+$ subset that might be considered a more powerful predictor of hematologic recovery than total CD34$^+$ cell count. Conversely, the number of CFU-GM reinfused should be considered the most powerful predictor of early unsupported-platelet recovery and duration of hospitalization (Table 4 and Figure 3a-b). Pecora *et al.* recently demonstrated in a heterogeneous series of 420 consecutive cancer patients, that the percentage of CD34$^+$/33$^-$ cells was a more reliable predictor of hematologic recovery than the total number of CD34$^+$ cells and concluded that the repopulating capacity of CD34$^+$ cells resides in the CD34$^+$/33$^-$ subset, whereas CD34$^+$/33$^+$ cells do not significantly influence early hematopoietic recovery.[20] We attempted to divide our series of patients according to the percentage of CD34$^+$/33$^-$ cells, using 50% as a cut-off (23 vs 19 patients): the Kaplan-Meier curves of neutrophil recovery to 500/µL and platelet recovery to 50,000/µL were identical in the two groups, as were the median values (data not shown). Therefore, our data did not confirm this hypothesis, although in univariate analysis the CD34$^+$/CD33$^-$ cell count appeared strongly

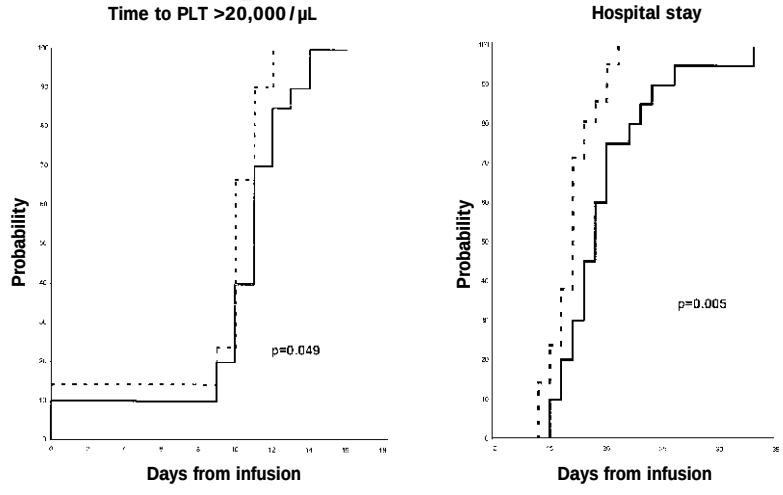

Figure 3. CFU-GM ($\times 10^4$/kg/bw) and Kaplan-Meier probability of a) time to unsupported platelet recovery to 20,000/µL; b) days of hospital stay. $\geq$ 136.2$\times 10^4$ CFU-GM/kg/bw (– – –), < 136.2 $\times 10^4$ CFU-GM/kg/bw (——).

Table 4. Multivariate analyses: significant results.

	Variable	Dichotomous			Continuous		
		Parameter estimate	P value	Risk ratio	Parameter estimate	P value	Risk ratio
Time to ANC > 500/µL	CD34+	0.878	0.02	2.4	0.063	0.04	1.06
Time to ANC > 1000/µL	CD34+	0.917	0.01	2.5	0.08	0.01	1.08
Time to PLT > 20,000/µL	CFU-GM	0.738	0.04	2.1			
	CD34+				0.136	0.002	1.14
Time to PLT > 50,000/µL	CD34+	1.027	0.008	2.8	0.15	0.0001	1.16
	CD34+/38-	0.712	0.04	2.03			
Hospital stay	CFU-GM	0.88	0.016	2.4	0.005	0.0004	1.006

correlated with the time to neutrophil and unsupported platelet recovery (Table 2). CD34+ cells can be divided into functionally distinct progenitor populations based on the presence of the CD33 antigen, that is expressed only by more differentiated committed colony-forming progenitors.[21,22] Experimental studies on rodents[23] and available clinical data from humans[24] indicate that transplantation is followed by two phases of engraftment associated with hematopoietic progenitors at different stage of maturation. In accordance with this biphasic model, Siena *et al*. have already reported that early hematopoietic recovery was better predicted by the number of CD34+/33+ cells, while CD34+/33- cells may be responsible for a second sustained engraftment phase.[10] On the other hand, correlation analysis in our series demonstrated that each CD34+ cell subset was strongly correlated with the total number of CD34+ cells and for this reason, it is unlikely that individual subsets could be considered as independent predictors of engraftment kinetics in multivariate analysis. In the Cox analysis, using variables dichotomized according to their median value, CFU-GM assays seemed to be the most powerful predictor of the time to unsupported platelet recovery to 50,000/µL, whereas when the data were considered as continuous variables, the total dose of CD34+ cells reinfused was confirmed to be the most reliable predictor of unsupported platelet recovery as well. Many other investigators have reported a good correlation between CFU-GM and neutrophil recovery, but not everyone has shown a strong correlation between CFU-GM and platelet recovery.[25-28] This is not surprising because CFU-GM are believed to reflect more committed myeloid progenitor cell activity. Anyway, our data should not be regarded in conflict, considering the strong relationship between CD34+ cell count and CFU-GM (r=0.7, *p*=0.0001). Furthermore, the number of CFU-GM reinfused, evaluated both as a dichotomous and a continuous variable, resulted to be the most useful indicator of duration of hospitalization (Table 4).

Our study attempted to address a couple of questions. The first was: is more really better? In our series of breast cancer patients treated with high doses of thiotepa and melphalan the answer is yes, since there was a strong correlation between the number of CD34+ cells reinfused and the engraftment kinetics. The second question dealt with the role of CD34+ cell subsets in predicting hematologic recovery and duration of hospital stay. In our setting of patients and, we can presume, for the majority of patients treated with high-dose chemotherapy without TBI and rescued with autologous PBPC, the engraftment success was nearly 100%. Therefore, the main end point must be the earlier hematopoietic reconstitution, and for that, the numbers and percentages of the different CD34+ cell subsets considered in our analysis had no advantage over the total number of CD34+ cells infused.

Contributions and Acknowledgments

CD and AC share main responsibility for all aspects of the study and for writing the paper. GR, GF and MM contributed to critical revision of the manuscript. ES was in charge of the statistical analysis, GM, MB, LZ and EF were responsible for the evaluation of cytofluorimetric analyses, harvesting , freezing and reinfusion of PBPC. All authors: final approval of the definitive version. Thanks to Dr. Piero Foglia for his kind revision of the manuscript, and to the nurses of the Oncology-Hematology Department for their continuous and skilled care of the patients. The order of the authors reflects their contribution to the study.

Funding

Supported in part by "High dose chemotherapy research project" Istituto Oncologico Romagnolo, Italy.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

Manuscript received August 25, 1999; accepted December 30, 1999.

Potential implications for clinical practice

- There is not any additional role for the CD34+ cells subsets considered in our analysis, compared with the total number of CD34+ cells in predicting the hematologic recovery and the duration of hospital stay.
- We confirmed that there is a strong correlation between the number of CD34+ cells and the engraftment kinetics.

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